THE EFFECTS OF 17-HYDROXYCORTICOSTERONE (COMPOUND F) ON HUMAN EOSINOPHILS

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(Accepted for publication 15th March, 1954.)

The factors which influence the disappearance of eosinophil leucocytes from the peripheral blood following the administration of corticotrophin (ACTH), 11-dehydro-17-hydroxycorticosterone (cortisone), and 17-hydroxycorticosterone (compound F), are not understood. One of the possible explanations of this phenomenon is that the active adrenal steroid causes lysis of these cells. To test this hypothesis blood was incubated for varying times with different concentrations of compound F. Under the conditions of these experiments there is no evidence to substantiate the claims that lysis is responsible for the phenomenon of hormone-induced eosinopenia.

Materials and Methods.

Venous blood from healthy persons was used, in which the number of eosinophils ranged from 40 to 400 per mm^3. Coagulation was prevented either by the addition of heparin or by defibrination.

The active adrenal steroid used was 17-hydroxycorticosterone (free alcohol).[2] This was prepared in two different concentrations: 33 μg. per ml. and 500 μg. per ml. in "glucose saline" containing 0·7 gm. NaCl, 0·6 gm. glucose in 100 ml. distilled water. At the concentration of 500 μg./ml. the steroid remained in fine suspension, but at 33 μg./ml. was in solution. 0·9 ml. of blood was mixed with 0·1 ml. of compound F to give a final concentration of compound F of 50 and 3·3 μg. per ml. The mixing and subsequent incubation took place in test tubes which had been treated with silicone (GE "Dri-film"). A tube was also used as a control in which 0·1 ml. of glucose saline was mixed with 0·9 ml. of blood. After the blood and compound F had been thoroughly mixed, duplicate samples were taken for eosinophil counts. The tubes were then incubated at between 37° and 38° C. for 5–6 hours, during which time they were stirred with siliconed glass rods. At the end of this period the numbers of eosinophils were again counted. The diluting fluid used for counts was a modification (Pilot, 1950) of that described by Randolph (1944). The samples were diluted 1 in 20 with a fluid that contained 0·05 p.c. phloxine in an alkaline propylene glycol solution. The pipettes stood for at least 20 minutes, and were then shaken mechanically for two minutes, and the number of eosinophils estimated by counting the cells in both sides of a Fuchs Rosenthal counting chamber (0·2 mm. deep). Films of the blood were made in some of the experiments before and after incubation. These were stained for 15 minutes by Giemsa, diluted 1 in 40 with distilled water.

[1]This investigation has been carried out in partial fulfilment of the requirements of the University of Melbourne for the Degree of Doctor of Philosophy.

[2]Kindly supplied by Dr. Elmer Alpert, Merck & Co. Inc., Rahway, N.J.

TABLE 1.

Changes (p.c.) in eosinophil counts at end of 4–6 hours' incubation. Heparinized blood. Ten experiments.

Statistical data	Control	Compound F 3·33 μg./ml.	Compound F 50 μg./ml.
Mean	+0·87	+1·14	−0·67
Standard error	2·12	1·97	2·82
Fiducial limits P=0·05	−3·85 to +5·59	−3·25 to +5·53	−6·95 to +5·61

TABLE 2.

Changes (p.c.) in eosinophil counts at the end of 4–6 hours' incubation. Defibrinated blood. Twelve experiments.

Statistical data	Control	Compound F 3·33 μg./ml.	Compound F 50 μg./ml.
Mean	−4·5	−2·84	−1·92
Standard error	3·17	2·91	3·04
Fiducial limits P=0·05	−11·41 to +2·41	−7·18 to +3·50	−8·54 to +4·70

RESULTS.

These are shown in Tables 1 and 2. It will be seen that there is very little change in the eosinophil counts after 4–6 hours' incubation. The differences between these changes are not significant at the 5 p.c. level. The results of the individual counts at 4, 5 and 6 hours have been pooled, since there is no consistent difference at these various times.

DISCUSSION.

In addition to causing a disappearance of circulating eosinophils, adrenal steroids cause a fall in the number of circulating lymphocytes. Dougherty and White (1944), using rats, showed that this lymphopenia was most probably the result of lympholysis. This was confirmed by Feldman (1952) who, by the use of supravital stains, observed morphologic changes such as vacuolation, cytoplasmic bubbling and ultimate disintegration of lymphocytes and monocytes which had been exposed to the action of adrenocortical extracts. Frank and Dougherty (1953) incubated compound F with human blood and showed similar changes in stained blood films. Trowell (1953), by using tissue culture methods, showed that cortisone exerted a lethal action on lymphocytes in concentrations as low as 0·1 μg. per ml. This was measured by the numbers of dead (pyknotic) lymphocytes, as seen on stained film preparations taken from tissue culture. Robertson (1948) was, however, unable to demonstrate any change in the numbers of lymphocytes at the end of 8–12 hours, after these had been incubated with

adrenocortical extracts at concentrations varying from 1 in 500 to 1 in 10,000. As the amount of active adrenal steroid varies considerably in different adrenocortical extracts, and since Trowell used pure solutions of cortisone, it would seem reasonable to accept his results as showing that cortisone does cause death of lymphocytes. This mechanism may then be responsible for the disappearance of lymphocytes following the administration of cortisone.

With regard to eosinophils, Baldridge *et al.* (1951), reported that cortisone in high concentrations (50 and 5 μg. per ml.) caused no significant change in eosinophil numbers when incubated with heparinized blood samples. Meuhrcke *et al.* (1952), claimed that the reason for this failure of cortisone to produce the lysis of eosinophils was the fact that heparin antagonized the action of cortisone (Godlowski, 1951). They showed that when cortisone (330 μg. per ml.) was incubated for 4 hours with blood which had been rendered incoagulable by defibrination, there was a significant reduction in the numbers of the eosinophils. They claim that morphologic changes can be observed in the eosinophils, but do not describe these in any detail. Godlowski (1952) also claims, from a small number of experiments, that the eosinopenic action of cortisone is by a direct lytic action. He states that "this action is seen in a blood film as a disintegration of the nuclei and cytoplasm of the eosinophils." No pictorial evidence is presented to support this statement. Cape *et al.* (1952), performed similar experiments in which they investigated the direct effect of compound F and cortisone at dose levels varying from 0·69 to 200 μg. per ml. of blood. They also used the plasma from patients who were under the influence of one or the other hormones, and whose eosinophils were maximally depressed. Neither incubation with the hormone nor plasma produced any change in the numbers of the eosinophils after 6–10 hours' incubation. Bibile (1953) reports a similar failure of both compound F and cortisone to cause lysis of rat and mouse eosinophils at dose levels of 2 and 5 μg. per 100 ml. of blood.

The results of the experiments outlined in this paper fail to confirm the initial report of Meuhrcke *et al.*, and of Godlowski, that the probable explanation of hormone-induced eosinopenia was a direct cytolytic effect of the hormones on the eosinophils. The dose levels in the experiments have been much higher than those which are associated with maximal eosinopenia which, according to Nelson and Samuels (1952), are of the order of 0·35 to 0·40 μg. per ml. The dose levels used in these experiments are of the order of 50 and 3·3 μg. per ml. These concentrations far exceed those found under pharmacological and certainly physiological, conditions. These are concentrations at which some eosinolytic effect should be apparent if this was the mechanism responsible for hormone induced eosinopenia. Nor did there appear to be any morphologic changes in the eosinophils at the end of 5–6 hours' incubation, as compared with the pretreatment samples; certainly none of the changes described by Godlowski or by Meuhrcke could be seen.

SUMMARY.

Blood was incubated with different concentrations of compound F for 4–6 hours at 38° C.

No significant changes were detected in the number of eosinophils in blood rendered incoagulable either by heparin or defibrination.

No morphologic changes were detected in the eosinophils.

The results of these experiments do not support the hypothesis that eosinolysis is responsible for the phenomenon of hormone induced eosinopenia.

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THE GROWTH OF HERPES SIMPLEX VIRUS

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(Accepted for publication 30th March, 1954.)

The growth of viruses in various host systems has generally been ascribed to the summation of a series of intracellular episodes. For any one infected cell, attachment of virus is followed by an eclipse phase during which infective virus may not be demonstrated in cellular extracts. This may be regarded as ending when infective virus again becomes detectable. Finally, infective virus is liberated either by explosion, as with bacterial viruses (Ellis and Delbrück, 1939), or by continued leak as in the case of many animal viruses (Henle, Henle and Rosenberg, 1947; Cairns, 1952).

Burnet (1954) has suggested that this pattern of multiplication may be confined to viruses and be characteristic of them. Because the sequence of events occurring during the growth of herpes simplex virus had not been determined, a study was made of the mode of multiplication of this virus in the chorio-allantoic membranes of intact chick embryos and in the membranes of de-embryonated eggs (Bernkopf, 1949). Since this work was completed, a similar account has been published by Scott, Coriell, Blank and Gray (1953), and it is of interest to compare their results with those presented here.

Materials and Methods.

Viruses.

Herpes simplex virus. The strain HFE, adapted in growth on the chorioallantois (Burnet and Lush, 1939a), was used in the form of a suspension of infected chorio-allantoic membranes. This was made by grinding with alundum and a small volume of nutrient broth (Wright, 1933). After centrifugation, the clear supernatant was mixed with an equal volume of sterile glycerol and stored at −20° C.

Influenza virus. The strain used was WSE influenza type A, which was adapted to chorio-allantoic growth by Burnet and Lush (1938) and recently maintained by allantoic passage. Suspensions of virus were used in the form of freshly harvested allantoic fluids from eggs inoculated by the allantoic route 48 hours previously, at the 11th day of incubation.

Enumeration of infective virus.

Virus was recovered from membranes by homogenizing without the use of abrasives. For this purpose, either the apparatus described by Anderson (1954) or a glass mortar with a

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closely fitting mechanically rotated pestle were employed. Both methods had been shown not to reduce the infectivity titres of viral suspensions, and at the same time gave good cellular disruption. They had, in addition, the advantage that the volume of the material could be assessed simply by using a graduated pipette, so that the total infectivity of the material could be measured.

Both viruses were titrated on the chorio-allantoic membrane by the method of Burnet and Lush (1939a). Serial ten-fold dilutions of virus were made in nutrient broth and 0·05 ml. of each inoculated onto the dropped chorio-allantois of eggs which had been incubated for 11–12 days. After incubation at 35° C. for 48 hours, the lesions were counted using the scheme outlined by Beveridge and Burnet (1946). The fewest eggs used for each dilution were four, allowing an accuracy of ±50 p.c. Only counts which averaged 5–50 lesions per membrane were accepted and the results expressed as infective units per ml. or total number of infective units per preparation.

The infective unit (i.u.) is defined as that quantity of virus which gives rise to one lesion on the chorio-allantois.

Preparation of de-embryonated eggs.

Eggs at the 15th or 16th day of incubation were de-embryonated, leaving *in situ* the chorio-allantois and as much of the allantois and amnion as possible (Burnet and Lind, 1954). They were washed thrice with physiological saline and drained well. The bathing fluid consisted of Ringer's solution containing 0·1 p.c. glucose, 50 μg./ml. streptomycin HCl (Glaxo) and 12·5 units/ml. calcium penicillin (Commonwealth Serum Laboratories). Five ml. of this fluid was put into each egg and the opening covered with an aluminium cap.

The eggs were set up on a turntable set at 75° from horizontal which revolved at 2 r.p.m. in an incubator held at 37° C. and 50–100 p.c. humidity.

Neutralizing antiserum.

Specific neutralizing antisera were prepared against HFE by inoculating young adult rabbits with three graded intradermal doses at weekly intervals: (i) 0·1 ml. formolized virus; (ii) 0·1 ml. virus suspension containing 2×10^3 i.u.; (iii) 0·1 ml. virus suspension containing 2×10^5 i.u. The formolized suspension was prepared by incubating a suspension containing 5×10^6 i.u./ml. with 0·01 p.c. formalin at 37° C. for 18 hours.

The first injection gave rise to small local ulcers which healed in 4–5 days. Thereafter the animals were apparently well until 3–5 days after the second injection when they again developed local lesions, failed to eat their food, and one developed a transient weakness of the hind limbs.

Four weeks after the first injection, the animals' sera neutralized 100 i.u./HFE by the method of Burnet and Lush (1939b).

Receptor destroying enzyme.

RDE was used in the purified form routinely employed in this Institute (Ada and French, 1950).

RESULTS.

The original intention of this work was to examine the growth of herpes virus in the de-embryonated egg and to compare it with that occurring in the chorio-allantois of the intact embryo. The chief virtue of the de-embryonated egg was thought to lie in the possibility of estimating the viral content of the whole system at any desired time. Additional advantages appeared to be offered by the ease with which washing or other manipulations might be performed.

It was found that whilst multiplication of herpes virus readily occurred, many technical difficulties arose with this system. Although extensive studies were made with de-embryonated eggs, covering all aspects of multiplication, relatively little use is made of the results here.

The principal disadvantages of the de-embryonated egg lay in the fact that a considerable proportion of virus always remained free in the large volume of bathing fluid used. Even after thorough washing of the preparation, virus was still found free in the fluids at a concentration which suggested that the virus is initially at least reversibly adsorbed to the membranes. Also, the thermal degradation of virus suspended in such fluid was considerable (0·32 $\log_{10}$ per hour). Moreover, substitution of broth, 1:10 horse serum saline or bathing fluids which had been taken from uninfected eggs at intervals only slightly delayed the rate of deterioration. It is obvious that little reliance may be placed upon results obtained with these preparations, especially in the early stages of multiplication. Again, growth in the de-embryonated egg may be limited by exhaustion of nutrients. The concentration of glucose was shown to limit the yield of virus. When 10^5 i.u. HFE were inoculated into de-embryonated eggs with 5 ml. broth and 0, 0·1 and 1·0 p.c. glucose, they were found to contain, respectively, $10^{5.6}$, $10^{6.0}$ and $10^{8.4}$ i.u. virus after 48 hours. A similar finding was made with influenza virus by Daniels, Eaton and Perry (1952). On the other hand, the de-embryonated egg maintains the pH of its contents within reasonable limits over a considerable period. A number of de-embryonated eggs were set up, each containing 5·0 ml. bathing fluid with no viral inoculum. Using a glass electrode, pH determinations were made at 15 intervals over a period of 36 hours. Apart from the values pH 6·83 and pH 7·95 at the 6th and 14th hours, the remaining fluids gave values which lay between pH 7·08 and pH 7·80.

One special advantage of the chorio-allantoically infected entire embryos was that, because herpes virus gives rise to lesions on the membrane, the system may be made self recording. By exploiting this quality, a valuable method of following the initial attachment of virus has been developed.

Initial attachment of virus.

Attachment of virus to a susceptible cell is an essential preliminary to infection. Information as to the nature and temporal features of this attachment process may be sought along three lines:

(1) Demonstration by chemical or enzymic methods of a specific receptor mechanism on the cell surface analogous to that which operates in influenza infections.

(2) Determination of the proportion of virus which may be removed from the inoculated system by washing (or its equivalent) at intervals after the addition of virus.

(3) Determination of the rate at which the viral elements, which are necessary for the formation of specific lesions, become inaccessible to neutralizing serum.

All of these approaches were used in the present work.

It is well established that there exists a specific adsorption mechanism for the influenza group of viruses which may be destroyed by the receptor destroying enzyme of *Vibrio cholerae* (Stone, 1948). It was expected that HFE would not be adsorbed by this mechanism, and this expectation was verified by the following experiment. The membranes of a number of eggs were dropped and 0·05 ml. amounts of purified RDE containing 500 units were placed in half the eggs. A similar quantity of broth was placed in the remainder. After incubation at 37° C. for one hour, small triangular windows were cut over the artificial air-space and 0·05 ml. of suitable dilutions of either HFE or WSE viruses were inoculated directly onto the membranes. The windows were covered with cellophane tape, the eggs incubated for a further 48 hours at 35° C., and the membranes then examined for lesions. Table 1 gives the results of such an experiment. It may be seen that whereas the number of lesions caused by WSE was markedly reduced by treatment with the enzyme, HFE gave rise to similar numbers of lesions on treated and untreated membranes, indicating that the influenza receptor mechanism is probably unnecessary for the adsorption of herpes.

TABLE 1.

The effect of pretreatment of the chorio-allantoic membrane with RDE upon the development of lesions by the viruses of influenza type A (WSE) and herpes simplex (HFE). Figures indicate the average number of lesions developing on four membranes.

	HFE	WSE
Untreated membranes	32	43
RDE treated membranes (1 hour 37° C.)	48	2

The rate at which HFE becomes immobilized on the chorionic surface was investigated by inoculating a number of eggs each with the same amount of virus (100 i.u.) in a total volume of 0·1 ml. broth. At intervals, eight eggs at a time were taken from the incubator and 0·05 ml. added to the volume of fluid already on the chorion. The hole in the shell was then sealed with paraffin and the eggs were candled to define the extent of the artificial air-space, which was marked on the shell. Each egg was then rotated through 120° about its long axis and the bubble of air was seen to force its way between the shell membrane and chorio-allantois to find a new position over a fresh area of that membrane. Any free virus present was carried over with the bubble of air in the small

volume of fluid bathing the chorion. The new position of the air-space was defined and a small hole drilled over it to prevent adsorption of the air within it. The eggs were returned to the incubator for a further period of 48 hours.

When the eggs were opened, the entire chorio-allantoic membranes were removed and examined. It was found that well defined lesions were formed whether the membrane had been pressing against the shell membrane or not, and it was a simple matter to define the extent of the original area of membrane inoculated. Preliminary experiments using this technique showed evidence of very rapid fixation of virus followed by an unexpected increase in the proportion of moveable virus between the 2nd and 3rd hours. This was apparently due to changes in the condition of the membranes after they had been dropped, for when the eggs were inoculated four hours after the membranes had been dropped, the effect disappeared. For this reason, with the experiments detailed in Fig. 1A, the membranes were dropped four hours before inoculation.

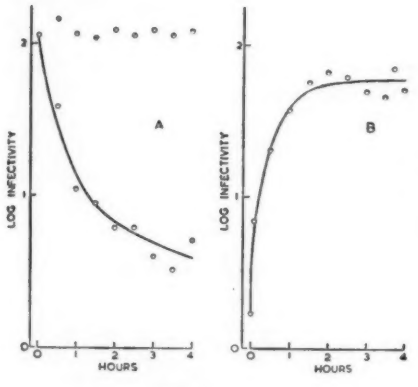

Fig. 1.

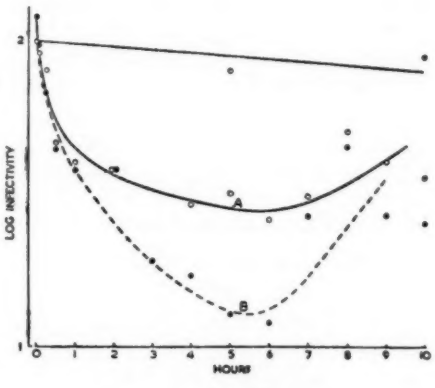

Fig. 2.

Fig. 1. A. Showing the rate at which herpes virus was immobilized on the chorio-allantois of entire chick embryos. The curve indicated represents the amount of virus (log_{10} i.u.) which was freely moveable at different times. The total virus for each determination is shown at the top of the figure. (For details see the text.)

B. Showing the rate at which herpes virus penetrates the cells of the chorio-allantois of entire embryos. The curve indicates the number of lesions (log_{10}) which developed membranes when 0·1 ml. neutralizing serum was added at intervals after inoculation with 100 i.u. virus. (For details see the text.)

Fig. 2. The eclipse phase. Showing the rate at which herpes virus becomes non-infective when inoculated on to susceptible cells.

Curve A was obtained when 100 i.u. virus were placed on the chorio-allantoic membranes of entire embryos, and the membranes titrated at intervals. The rate of thermal degradation is shown at the top of the figure.

Curve B was obtained using de-embryonated eggs which contained 0·1 ml. bathing fluid. At intervals, the membranes were homogenized in the bathing fluids and titrated for infective virus. (For details see the text.)

The curves shown relate to total virus at any one time (total lesions) and to free virus (lesions found away from the original area of inoculation). It may be seen that the total count remained constant throughout the experiment, but that the virus was rapidly immobilized on the original site of inoculation. About 90 p.c. of the virus became fixed within the first 90 minutes, after which the curve flattens out. A small proportion of the virus remained free even at the fourth hour.

The proportion of infective particles which had at a particular time become inaccessible to neutralization by serum was investigated, using the method employed by Burnet and Lush (1939a) and by Shaffer and Enders (1939). A number of eggs were inoculated with about 100 i.u. virus, and at intervals during incubation at 35° C., 0·1 ml. undiluted specific neutralizing serum per egg was added to a group of eight eggs. After a total incubation period of 48 hours, the membranes were removed and examined. In Fig. 1B the results of such an experiment are shown. It may be noted that the curve flattens out after 90 minutes as in the previous experiment, but by contrast only 35 p.c. of the virus had apparently become inaccessible to serum by this time. This suggests that a stage may exist in which proportion of the virus immobilized on the susceptible cells remains accessible to neutralizing serum. It must be pointed out that a number of lesions counted as primary lesions may, in fact, be initiated later in the course of growth. These would not be expected to develop in the presence of neutralizing serum.

Evidence of an eclipse phase.

There are serious difficulties in the way of demonstrating unequivocally the existance of an eclipse phase during the course of multiplication of any animal virus. The standard approach is to attempt to demonstrate a significant fall in the titre of infective virus during the first few hours after infection. Such an experiment measures only the proportion of virus which remains fully infective after undergoing the particular conditions imposed upon it.

A fall in the infectivity titre may result from:

(1) Removal of the virus particle to some portion of the host which is not included in the material titrated.

(2) Inactivation by thermal degradation or some incidental cellular activity which is not concerned with the initiation of infection.

(3) Transformation of infective virus to a form that is not invasive (or whose infectivity cannot survive the techniques used for tissue disintegration) as a necessary step in viral multiplication.

The last is the only phenomenon which should be considered as an eclipse phase and, therefore, any investigation must establish that this is the cause for the observed diminution in infectivity.

In the experiments with the chorio-allantoically inoculated eggs, the first technical difficulty encountered was to prevent the fluid lying on the surface of the membrane from running off and mixing with the allantoic fluids when the membranes were being excised. For this reason, the recovery of virus during the first few hours after inoculation was lower than it should have been. This difficulty was overcome by inoculating virus suspended in broth containing 5 p.c. gelatin. Preliminary experiments showed that at this concentration of gelatin the inoculum was a freely mobile fluid at 35° C., which gelled in 20 minutes when an egg inoculated with 0·05 ml. was transferred to the coolest part of the refrigerator.

Curve A in Fig. 2 shows the rate at which the infectivity of HFE diminished when a number of eggs inoculated onto the chorio-allantois with 100 i.u. were titrated for infective virus. At intervals, the eggs were transferred to the coolest part of the refrigerator, left to chill for 30 minutes, and the membranes carefully excised together with the adherent jelly. After homogenization, the material was titrated for infectivity using eight eggs for each dilution. It is clear that there was a steady fall in the titre which flattened out after the fourth hour. It was reasonably certain that the fall observed was not due to thermal degradation because a control tube of the inoculum in gelatin maintained its titre at 35° C. throughout the experiment, and it was easy to ensure that all the jellified inocula were recovered when the membranes were harvested. It is probable that the rate of eclipse of virus was delayed by the increased viscosity of the inoculated fluid which contained gelatin, because when the "turning experiment," outlined above, was repeated with and without 5 p.c. gelatin as the suspending medium, it was found that after 30 minutes 29 p.c. of the virus remained moveable with broth alone, but 49 p.c. in the case of gelatin broth. Again, after 2 hours, the figures were respectively 10 p.c. and 21 p.c. Because of this delayed attachment to the host cells, it follows that the slope of Curve A in Fig. 2 might have been steeper if the gelatin had been omitted.

Additional evidence of viral eclipse was obtained using de-embryonated eggs. In order to reduce the quantity of virus remaining in the bathing fluid to a minimum, the volume of bathing fluid used was reduced fifty-fold to 0·1 ml. Whilst this must have reduced the efficiency of the preparation, the curve obtained by titrating the infectivity of the entire membrane-fluid system at intervals (Curve B, Fig. 2), is remarkably similar to that obtained using entire chorio-allantoically infected eggs. It is likely that the two curves shown in Fig. 2 are the same. It has been pointed out that the Curve A probably falls too slowly because of the increased viscosity of the gelatin broth. The slope of Curve B is, on the other hand, likely to fall too rapidly because it is probable that losses due to thermal degradation would be greater in this experiment.

The similarity of the results obtained with these two types of experiments strongly suggest the occurrence of an eclipse phase. Unequivocal evidence was

not, however, obtained until an experiment was devised similar to that used in the study of the growth of Meningo-pneumonitis virus by Girardi, Allen and Sigel (1952). Four de-embryonated eggs were inoculated with HFE and the inoculum titrated for infectivity. After incubation on the turntable for 120 minutes, the eggs were three times washed out with Ringer's solution and the bathing fluids replaced with 5·0 ml. 1:4 specific neutralizing serum in Ringer's solution. The eggs were incubated for a further period and at the fourth hour of the experiment the eggs were removed. The membranes were harvested after washing the eggs out five times with physiological saline. Each was cut into two equal portions, one of which was again divided into 18 pieces. Of the 72 slices obtained in this way, 36 were chosen at random and each inoculated onto the dropped chorio-allantois of an egg at the 11th day of incubation. The remaining half membranes were pooled and homogenized, 0·05 ml. of this material being inoculated onto the chorio-allantois of 18 eggs. After incubation at 35° C. for 48 hours, the eggs were examined. It was found that only two lesions had developed in the eggs inoculated with the homogenate. All the slices inoculated had apparently become grafted to the chorio-allantois upon which they were inoculated, and in four instances a number of characteristic herpetic lesions had developed around the grafts. The remaining membranes were ground up and sub-inoculated onto other eggs which were incubated for a further 48 hours. In 21 instances lesions resulted from these sub-inoculations. Thus, 25 out of 36 slices contained active virus which subsequently became infective. It follows then that at least 25 potential i.u. of active virus were present in 36 slices. These results are presented in Table 2. It may be seen that at the fourth hour of the experiment more than 10 times as much virus was demonstrable by the grafting method as was detected as infective virus.

TABLE 2.

Showing the relative quantities of infective virus and virus capable of multiplication in the membranes of de-embryonated eggs 4 hours after inoculation with 200 i.u. herpes virus per egg. Values refer to infective virus (actual or potential) per egg.

Virus inoculated	Virus recovered	
	Infective virus (homogenate)	Actively multiplying virus (slices)
200	2·4	25

Maturation and release of virus.

In the subsequent section, the results are given of the maturation and release of HFE. Maturation is defined as the appearance of new infective virus in the homogenized infected membranes. The terms liberation or release are applied to the appearance of infective virus in the fluids bathing the membranes in de-

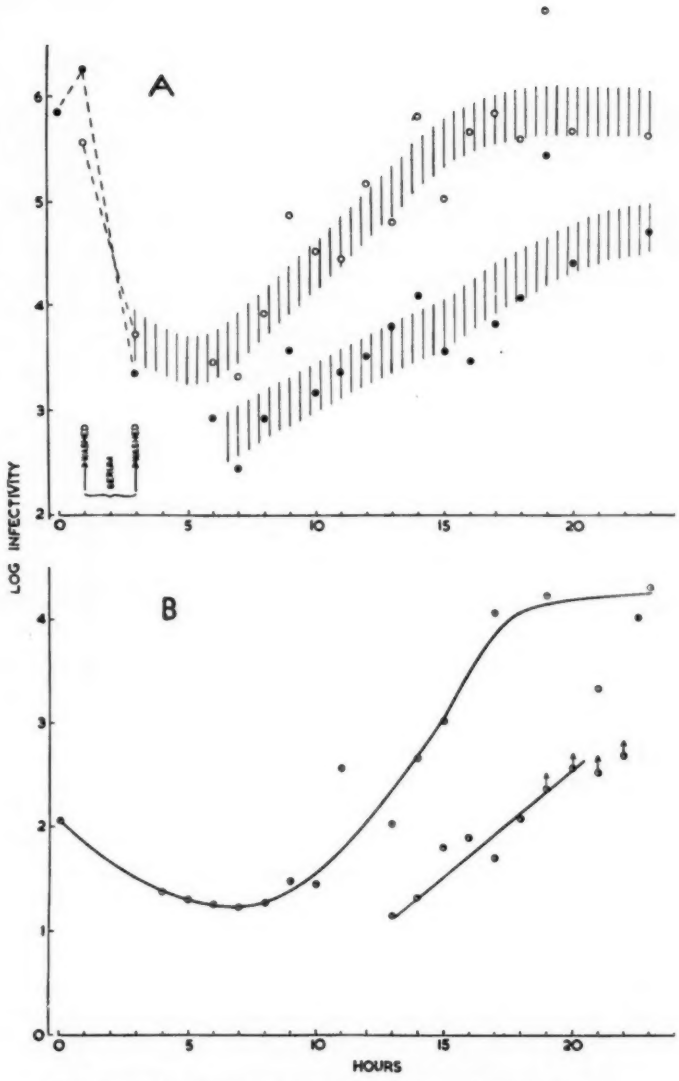

Fig. 3. Showing the maturation and release of herpes virus.

A. De-embryonated eggs were each inoculated with 6·95 $\log_{10}$ i.u. virus. The eggs were washed out after 1 hour and filled with 1:10 neutralizing serum. At the 3rd hour the eggs were again washed and replenished with 5·0 ml. bathing fluid. Thereafter the eggs were harvested and the membranes and fluids separately titrated hourly.

○ Virus in the membranes. ● Virus in the fluids.

B. Entire embryos were inoculated by the chorio-allantoic route with 100 i.u. virus. At intervals the chorio-allantoic membranes were titrated for infective virus. Between the 13th and 22nd hours the release of virus was followed by the "turning experiment" (see the text for details).

⊙ Virus in the membranes. ◑ Virus in the fluids.

embryonated eggs and in the washings of the chorio-allantois. The experiments already reported make it clear that before satisfactory results may be obtained from experiments using de-embryonated eggs, it is necessary to eliminate residual virus in the bathing fluid. A satisfactory device, which has been shown not to interfere with viral growth, was to wash out the eggs thrice after allowing one hour for virus to become attached and to penetrate host cells. Then 5·0 ml. 1:10 neutralizing serum was introduced and, after incubation for a further two hours, the serum was washed out and replaced by 5·0 ml. glucose-antibiotic-Ringer solution. From the third hour onwards the membranes and the fluids were titrated separately at intervals. The results of such an experiment are given in Fig. 3A.

When the gross architecture of the curves are examined, it is clear that the content of infective virus in the membranes increased logarithmically between the 6th and 19th hours.

This represents a total increase of about 2·5 log:. Simultaneously, that of the fluids increased by 2·0 log:. However, there is a suggestion that three peaks occurred in the curves of both membranes and fluids at the 9th, 14th and 19th hours. The significance of these is discussed later. It is of interest that the infective virus in the fluids follows but does not exactly reflect that in the membranes. This suggests that a part of the virus leaked out of the cells as it matured. However, at the 19th hour there was a suggestion that the release of virus took place more rapidly. This apparent burst of liberated virus has been observed upon several occasions in this type of experiment.

The main features of the two curves were confirmed using the dropped chorio-allantoic membrane technique. About 100 i.u. HFE in 0·05 ml. was inoculated per egg. At intervals between the 13th and the 22nd hours the infectivity of some membranes was titrated. Other eggs were "turned" after adding 0·1 ml. broth in the same way as was described for the experiment which dealt with the rate of immobilization of virus. It may be seen in Fig. 3B, that the total content of infective virus in the membranes increased logarithmically by 3·0 $\log_{10}$, the maximum being found at the 19th–20th hour. Again, the liberation of virus, as shown by the "turning experiment," followed the maturation, but did not parallel it. Although fewer points were determined, there was no suggestion of peaks occurring at about the 14th hour as in the previous experiment. Unfortunately, the counting of lesions due to liberated virus after the 18th hour became impossible owing to their multitude so that these counts were most inaccurate, erring on the low side. It is therefore not possible to determine whether there was a more massive release of virus at or after the 19th hour. An incidental observation made during this experiment was that at the 22nd hour, and thereafter, small but definite lesions were observed on the membranes which were harvested.

DISCUSSION.

Until the recent work of Scott *et al.* (1953), the eclipse phase of herpes virus multiplication had not been systematically studied. However, Burnet and Lush (1939c) had found that virus inoculated intracerebrally into mice temporarily disappeared and became detectable again within 24 hours. Shaffer and Enders (1939) had shown that 90 minutes after inoculation less than one-tenth of the original inoculum was demonstrable in the chorio-allantoic membrane. Again, Stulberg and Shapira (1953) had observed that herpes virus growing in tissue cultures had diminished in infectivity at 24 hours and subsequently increased sharply.

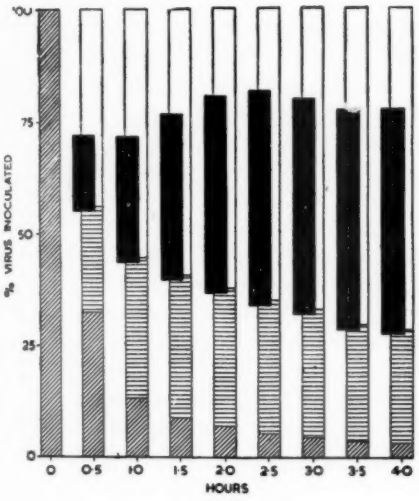

Fig. 4.

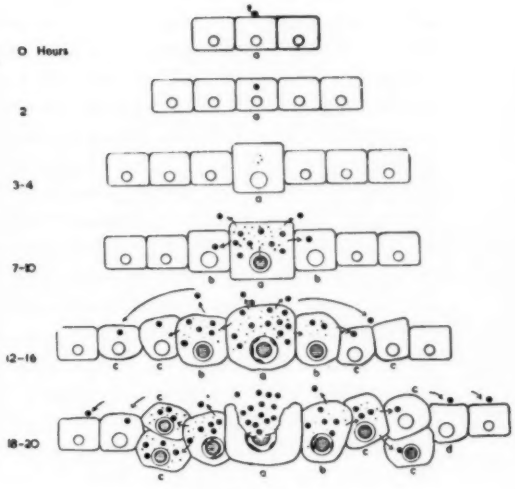

Fig. 5.

Fig. 4. A comparative histogram of the results shown in Figs. 1 and 2. The percentage of virus inoculated which remains infective at the intervals shown is indicated by the total shaded areas. Oblique hatching shows the percentage which has not become attached, and the transverse hatching the virus which has become immobilized but remains infective. The black columns represent the percentage of virus inoculated which had penetrated the cells, as demonstrated by the number of lesions which developed when neutralizing serum was added at intervals.

Fig. 5. An abbreviated scheme of the events occurring during the multiplication of one infective particle of herpes virus. During the first four hours the particle (black circle) penetrates the cell *a* and is transformed into a non-infective form (dotted). Replication of viral element occurs, and by the 7th to 10th hours inclusion formation begins (transverse hatching). From this time onwards virus matures and leaks away from the cell. Neighbouring cells (*b,b,c,c,c*) may be infected either directly or by transverse spread, or indirectly by way of the fluid bathing the cells. Between the 12th and 16th hours maturation and liberation of new virus continues from cell *a*. At this time new virus is maturing in secondarily infected cells (*b,b,c,c,c,c*) and is leaking away from them. Cellular proliferation occurs, increasing the number of available host cells. Eighteen to 20 hours after infection the original cell *a* becomes necrotic, discharging mature virus into the fluid bathing the cells.

The results of the present investigation are in very close agreement with those of Scott *et al.* (1953), and the two studies allow a fairly clear picture of the process of infection to be presented. In Fig. 4 the results of three experiments, which have been given in Figs. 1A, 1B and 2, are diagramatically compared. These three experiments were made using inocula of about 100 i.u. and under similar conditions. It should be borne in mind that the use of gelatin in the experiments showing the eclipse phase has probably distorted this curve.

The process by which virus particles enter susceptible cells appears to be divisible into three stages. There is first a period of reversible attachment in which the virus remains infective and may be neutralized by serum. During the next phase, one or other of these manifestations of viral activity is lost. The figure suggests that demonstrable infectivity is lost before the particle becomes inaccessible to serum. On the other hand, it was not found possible, using large inocula, to demonstrate total disappearance of infectivity even after very adequate treatment with 1:2 neutralizing serum. It is thus possible that a small proportion of virus entering cells remains infective and becomes inaccessible to serum. It is likely that the changes taking place in the virus which are measured by the two types of test are not identical. Finally, the particle enters the eclipse phase in which it is demonstrable as an active agent only by the potential virus and/or lesion-producing capacity of the intact host cell concerned.

Scott *et al.* (1953) obtained evidence that the attachment of herpes virus occurs more rapidly than our results suggest. By washing membranes at intervals, they found that more than 99 p.c. of virus had been taken up within the first hour as against 87 p.c. found here with the "turning experiment." Again, by titrating membranes which had been bathed in neutralizing serum for different periods, they found that a sharp rise in titre occurred when the serum had been added later than 10 or 15 minutes after inoculation, indicating that the penetration of cells took place very rapidly. These discrepancies may be due to the use, by Scott *et al.*, of a substrain of HF with a greater capacity for cell entry or to some unrecognized differences in technique. Experiments of a similar type by other workers have given results more in line with ours. Andrewes (1930), working with tissue cultures of rabbit testicle, found that serum would not prevent the formation of herpetic inclusions when added 30 minutes after infection. Burnet and Lush (1939a) and Schaffer and Enders (1939) both showed that the penetration of herpes virus took some time to complete. Their experiments using neutralizing serum were essentially like the one reported here and yielded similar results.

The initiation of infection by herpes virus has been described above. It is less easy to construct a clear picture of subsequent intracellular events. However, the results comprise three main findings: (1) During the period of maturation virus is formed logarithmically within the cell to reach a maximum representing approximately 100-fold increase at the 19th to 20th hour. (2) Virus

is liberated simultaneously with maturation but at a slower rate. There is a suggestion that liberation is increased as the 18th hour is approached. (3) Besides being released into the fluid bathing the infected cells, it is most probable that virus is transmitted directly to adjacent cells, thus initiating infection without entering the bathing fluid. This was suggested by the fact that, although neutralizing serum effectively prevented initiation of lesions, it did not alter the size or appearance of those where infection had already taken place. The same conclusion may be derived from the experiment in which Scott *et al.* (1953) demonstrated that substantial multiplication occurred in the presence of neutralizing serum. Considering the membranes as a whole, there was a suggestion that three episodes occurred in the de-embryonated eggs showing peaks at the 9th, 14th and 19th hours. It would be expected that the limited period allowed in this experiment for viral attachment would increase the likelihood of synchronizing intracellular cycles and thus favour the appearance of peaks in the infectivity curves. Whilst the peaks which occur at the 9th to 10th hours and 19th to 20th hours have repeatedly been demonstrated, the full experiment has not been repeated. The evidence suggesting that the peaks may represent a cyclic episode is that they are regular in time and, in the case of the membranes, in extent of increase. Also they occur simultaneously in the fluids.

A reconstruction of events has been presented in Fig. 5. Here the data have been fitted into a highly simplified diagram which attempts to relate the changes observed in the virus with those in the cells, namely, proliferation and formation of inclusions. One virus particle enters the host cell as described above. At the 3rd to 4th hour the eclipse phase occurs, and by the 7th to 10th hours maturation has begun and continues until the 18th to 20th hours. It may be seen that liberation of virus begins at the same time as maturation, virus being released into the bathing fluids and transversely. Thus cells are secondarily infected from the 7th to 10th hour onwards. At the 18th to 20th hour the original host cell becomes necrotic and bursts, liberating its contents. It is suggested that, for a time, the maturation of virus in secondarily infected cells is about equal to the release from necrotic cells so that little change is observed in the viral content of the membranes at this time. Maturation and release of virus from the secondarily infected cells begins at about the 14th hour, giving rise to the intermediate peak in Fig. 3A.

Superimposed upon and complicating this already intricate pattern is the proliferation of the infected host tissue. The lesion, which has become visible by the 22nd hour, at that time contains many cells which have proliferated. It is thought probable that the proliferation of host cells begins at the time of maturation, and it is fairly reasonable to suggest that it must play a part in viral multiplication because, unless the new cells are insusceptible to infection, their increased number must facilitate transverse spread of virus. Further light on this aspect has been shed by Crouse, Coriell, Blank and Scott (1950). Using

cytochemical tests, they showed that at the 10th hour after inoculation with 10^4 i.u., an occasional cell containing intranuclear inclusion could be made out. By the 18th to 20th hours definite plaques of cells could be seen in which 90 p.c. of the cells contained specific nuclear changes. The inclusions were seen to age gradually, losing their positive Feulgen reaction, and at the 30th hour a fresh crop developed.

It is emphasized that the rather diffuse picture of the events occurring in the multiplication of herpes virus, which has been described above, represents only the bare bones of what might occur. The steps which have been outlined as though they were irrevocable and irreversible are based upon a series of periodic static observations of a number of dynamic processes. It is known that not all the infective particles invade susceptible cells, and of those which do so a proportion probably go astray within the cell. Indeed, it is highly probable that incomplete virus may be formed in this way. Though it is evident that the initially attached virus may be washed away, it is not known how many other processes contributing towards multiplication are reversible.

SUMMARY.

The growth of the strain HFE of herpes simplex virus has been studied both on the chorio-allantois and in de-embryonated eggs. The virus grows readily in the latter preparations.

The evidence suggests that virus is rapidly immobilized onto susceptible cells. At first virus remains infective and is accessible to neutralizing serum on the surface of the membrane. Later, virus may not be removed by washing and is not accessible to serum. The influenza receptor mechanism plays no part in the infection of cells by HFE.

Virus becomes non-infective more slowly than it is attached. Whilst it is possible that this occurs as it enters the cell, there is a suggestion that it remains infective for a short period after it has become inaccessible to neutralizing serum bathing the cell surface.

Between the 8th and 20th hours infective virus is formed in an exponential manner, giving a total increase of about 100-fold. Virus is liberated into fluids bathing the cells as it matures. A part of the virus is transmitted directly to adjacent cells. These become secondarily infected, therefore, early in the course of maturation and liberation of virus.

Acknowledgments. It is a pleasure to thank Sir Macfarlane Burnet for his guidance, interest and suggestions during the course of this work; also to thank Dr. C. C. Curtain for making the pH determinations, Miss Lois Larkin for technical help, and Miss Mary Cook, who kindly drew the figures.

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THE COMPLEMENT FIXING ANTIGEN OF HERPES SIMPLEX VIRUS

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(Accepted for publication 30th March, 1954.)

In recent years evidence has accumulated suggesting that the complement fixing antigen of herpes simplex virus is a soluble component separable from the elementary particle (Hayward, 1949; Dudgeon, 1950; and Brown, 1953). Hoyle (1948) and Henle and Henle (1949) have shown that the soluble antigen of influenza virus can be detected before any rise in the haemagglutinin or infectivity titres occurs. Although the validity of the observation has been criticized by Fulton (1949) and others, it has provoked much interesting speculation upon the function of soluble antigens. This paper reports an investigation of the relationship between the complement fixing antigen and the elementary particle of herpes virus.

MATERIALS AND METHODS.

The strain of virus employed (HFE), the small scale methods of recovery, the titration of infectivity and egg techniques employed are all described in the previous paper (Wildy, 1954). The infective unit (i.u.) is defined as the amount of virus necessary to give rise to one lesion when inoculated onto the chorioallantoic membrane of an egg at the 11th day of incubation.

Preparation of large scale virus suspensions from membranes.

Because the apparatus used for homogenizing membranes would only accommodate a small volume of material, the membrane preparations employed for ultracentrifugation were prepared in a small Waring-type blender kindly made by Mr. M. Simpson. It consisted of a glass sweets jar 6×6×11 cm. high, of 300 ml. capacity, equipped with a screw cap in which was fitted a brass bush. Two blades mounted on a stainless steel shaft were driven at approximately 6,800 r.p.m. by a vertical electric drill. It was found that good disruption of membranes occurred if the material was homogenized when frozen into a solid block. Accordingly infected membranes were removed and pooled in 15×2·5 cm. test tubes which were stored overnight at −20° C. On the following morning, the tubes were held under a running tap until the frozen blocks of membranes could be removed intact. These were transferred to the blender without added diluent and the blades rotated for 10 to 15 minutes. The resultant fluid, which still contained some particulate material, was centrifuged at about 3,500 r.p.m. to remove larger particles. The supernatant obtained was then spun in the ultracentrifuge

[1]In receipt of the British Memorial Fellowship in Virology, Lecturer in Bacteriology, St. Thomas's Hospital Medical School, London. Supported by a grant from The Australian National Health and Medical Research Council.

at 2,400 *g.* for 20 minutes to clarify it. The final supernatant, which was shown to have retained its infectivity and complement fixing activity, was used as the starting material for ultracentrifuge studies. This is referred to as *clarified membrane suspension.*

Titration of complement fixing antigen.

Complement fixation tests were made following the general method of Hayward (1949), but using the drop as unit volume (Donnelley, 1951). Two-fold dilutions of the material to be tested were made in calcium magnesium saline containing 2½ minimal haemolytic doses (M.H.D.) of guinea-pig complement. An equal volume of inactivated human serum, which was known to contain herpes complement fixing antibody, was added to each tube. After holding at 4° C. for 18 to 20 hours, the tubes were allowed to regain room temperature and one volume of 2 p.c. washed sheep red cells, which had been sensitized with 3 MHD haemolysin, were added. The tubes were then incubated at 37° C. for 30 minutes and examined after they had stood on the bench for one hour. The endpoint taken was 50 p.c. haemolysis assessed visually.

Controls.

The following controls were invariably set up in parallel with the tests. Diluent, a set of complement dilutions, test material alone, test material with complement, test material with complement and a known negative serum and all the sera used both by themselves and with dilutions of complement. Anti-complementary material was occasionally encountered and, more seldom, non-specific reactions with negative serum. From time to time a standard antigen was titrated in parallel with the tests but, because all tests were compared only with others made at the same time and with identical reagents, this was not invariably done. Ground uninfected membranes were occasionally titrated, always with a negative result.

Sera.

The sera used were diluted 1:8 with calcium magnesium saline and inactivated at 62° C. for 15 minutes. This dilution was chosen because preliminary chessboard experiments had shown that for the sera used it was the optimal antibody dilution for all dilutions of antigen.

In each case material was titrated against two standard sera from the same two individuals. In many experiments, where there was sufficient material, the titrations were made in duplicate with each serum. The arithmetic mean of the separate titrations was taken as the final endpoint.

Accuracy of the method.

In order to obtain an estimate of the accuracy of this method, 24 parallel titrations for complement fixing antigen were made on a sample of clarified membrane suspension. The reciprocal titres obtained gave a mean value of 10·3 with a standard deviation of 3·5 for any one determination.

The unit of complement fixing antigen.

In order to facilitate the comparison of complement fixing activity with infectivity, an arbitrary unit, the complement fixing unit (c.f.u.), is defined as the amount of complement fixing antigen (c.f.a.) necessary to give 50 p.c. haemolysis with 2½ MHD complement under the conditions of the test. Because the drop used in the tests was 0·04 ml., it follows that a titre of 1:4 is equivalent to 100 c.f.u. per ml.

Physical methods.

Sedimentation studies were made using an air-driven refrigerated ultracentrifuge whose rotor held twelve tubes at 10° to the vertical. Each tube was 12 mm. in diameter and held 8·3 ml. when full. The gravitational stress recorded is that at the centre of the axis of each tube. Centrifugation was carried out as near to 0° C. as possible.

RESULTS.

Comparison of c.f.a. and infectivity titres.

A comparison has been made of the content of c.f.a. and infective virus in different egg preparations which were incubated for varying periods after inoculation with HFE. Two experiments were made, in one of which entire embryos were used, and in the other de-embryonated eggs.

The experiment using entire embryos was carried out in the following way. Eggs were inoculated on four successive days with a constant inoculum and all harvested on the fifth day. Four eggs at a time were used and the materials derived from them pooled after harvesting. In one group of eggs virus was inoculated into the yolk-sacs on the eighth day of incubation and the amniotic and allantoic fluids harvested separately after varying periods of incubation at 35° C. A second group of eggs, which had been incubated for eleven days, were inoculated on the chorion and the chorioallantoic membranes were carefully excised and homogenized at intervals. Titrations for c.f.a. and infective virus were simultaneously made on all these materials.

TABLE 1.

Showing the relative infectivity titres (i.u. per ml.) and complement fixing titres (c.f.u. per ml.) of different preparations of HFE. The values given are all $\log_{10}$. The ratio infectivity/complement fixing activity of each preparation is given in italics.

	Chorioallantoic inoculation				Yolk sac inoculation						
		Chorioallantoic			Amniotic				Allantoic		
Material	Survival ratio	membranes i.u.	c.f.u.	ratio	Survival ratio	fluids i.u.	c.f.u.	ratio	fluids i.u.	c.f.u.	ratio
Inoculum (undiluted)	—	7·56	2·43	*5·13*							
24 hours	4/4	6·77	2·56	*4·21*	4/4	5·29	<1·70	—	5·51	<1·70	—
48 hours	4/4	7·88	3·60	*4·28*	3/4	7·73	2·02	*5·71*	6·16	<1·70	—
72 hours	4/4	8·68	3·51	*5·17*	0/4	8·32	2·42	*5·90*	7·34	2·30	*5·04*
96 hours	0/4	8·71	3·68	*5·03*	0/4	8·53	3·81	*4·72*	6·98	2·62	*4·36*

From the results, shown in Table 1, three facts are clear: (i) Complement fixing antigen can only be detected in preparations containing high concentrations of infective virus (circa 6×10^6 i.u. per ml.). (ii) The ratio i.u./c.f.u. is not a constant one, varying in different preparations by as much as fifty fold. (iii) The content of infective virus and of c.f.a. in amniotic and allantoic fluids continued rising after death of the embryos. Indeed, no c.f.a. was demonstrable in the allantoic fluids until after this had occurred.

With the experiment using de-embryonated eggs, the eggs were de-embryonated after incubation for 15 days. Inocula were prepared from the amniotic fluids of eggs which had been inoculated with HFE by way of the yolk-sac three

days previously (Nagler, 1946). Four pairs of eggs were inoculated with four serial dilutions of amniotic fluids, each egg receiving 5 ml. The inocula were left in the eggs for two hours, after which the residual free virus was removed and the eggs washed out with three rinses of saline. Five ml. glucose-antibiotic-Ringer's solution was then introduced into each egg. After incubation for twenty-four hours, membranes from one of each of the four pairs of eggs were harvested and titrated for infective virus and c.f.a. The fluid in the remainder was removed and replaced with fresh bathing fluid. These eggs were removed after a further period of 24 hours and their membranes titrated.

TABLE 2.

Showing the relative contents of infective virus (i.u.) and complement fixing antigen (c.f.u.) of de-embryonated eggs inoculated with various amounts of HFE at 20 and 44 hours. The ratio infectivity/complement fixing activity of each is given in italics. All values are $\log_{10}$.

Virus inoculated (i.u.)	6·98			4·98			2·98			0·98		
	i.u.	c.f.u.	ratio	i.u.	c.f.u.	ratio	i.u.	c.f.u.	ratio	i.u.	c.f.u.	ratio
20 hours	8·58	2·59	*5·99*	6·69	2·06	*4·63*	5·20	<1·86	—	3·56	<1·81	—
44 hours	7·80	2·85	*4·95*	6·97	2·28	*4·69*	6·75	2·46	*4·29*	4·65	<1·88	—

The results given in Table 2 confirm two findings of the previous experiment: (i) c.f.a. can only be detected in preparations containing high concentrations of virus and (ii), the ratio i.u./c.f.a. varies fifty fold in different preparations.

No inference may be drawn from the observation that c.f.a. can only be detected in the presence of high concentrations of infective virus, but it is obvious that this impedes any attempt to follow the time of its appearance in the growth cycle. The increase in the content of c.f.a. in the embryonic fluids after death of the embryo is almost certainly due to release of intracellular constituents by autolysis of cells. This is emphasized by the fact that no such increase was observed in the case of chorioallantoic membranes. The significant variation in the ratio between infective virus and complement fixing antigen indicates that the antigen is detected by the complement fixing test used here in a separate entity from infective virus.

Separation of c.f.a. from infective virus.

Evidence has been provided from three independent sources that the complement fixing antigen of herpes virus is a soluble component separable from infective virus (Hayward, 1949, Dudgeon, 1950, and Brown, 1953). On the other hand, under the conditions of testing employed by Fowler (1950), c.f.a. and infective virus could not be separated. The finding described above that the ratio i.u./c.f.a. is not a constant one suggests that two components exist, but does not indicate whether they may be separated.

Accordingly, using the ultracentrifuge, the separation of c.f.a. from infective virus was attempted in two ways, by sedimentation of infective virus, and by washing infective virus free of c.f.a.

Sedimentation of infective virus.

Infective virus was sedimented from suspensions by centrifugation at 6,000 *g.* for one hour. In order to obtain efficient separation of c.f.a. and infective virus, from each tube holding 8·3 ml. the top 1·2 ml. only was removed carefully with a pasteur pipette. These portions were then pooled and spun at 6,000 *g.* for one hour and the top 1·2 ml. again carefully removed. In each case, after removing the top 1·2 ml., the deposit was resuspended in the volume of fluid remaining in the tubes. The volumes of the samples were measured and titrations were made for infective virus and c.f.a. Four experiments of this type were made. In two of these, amniotic fluid was used which had been harvested three days after inoculation of HFE into the yolk sacs of eight-day old eggs (Nagler, 1946). The other experiments were made on clarified membrane suspensions which had been derived in one case from eggs inoculated 48 hours previously on the chorioallantois at the eleventh day of incubation, and in the other from de-embryonated eggs which had been incubated for 22 hours.

TABLE 3.

Showing the separation of c.f.a. from infective virus by sedimentation of the infective particle.
** 1 ml. samples were removed before the materials were again spun. All values are given as log_{10} units.*

Material	Sample	Volume (ml.)	c.f.u.	Recovery (p.c.)	i.u.	Recovery (p.c.)
(i) Amniotic fluid	Original	40·0	2·40		8·42	
	1st top portion	8·5*	2·22	—	7·02	—
	2nd top portion	1·2	2·24		5·95	
(ii) Amniotic fluid	Original	40·0	2·30		8·42	
	1st top portion	8·5*	2·15	110	7·13	62
	1st lower portion	31·5	2·40		8·10	
	2nd top portion	1·2	2·10	113	6·34	103
	2nd lower portion	6·3	2·23		7·20	
(iii) Clarified membrane suspension (de-embryonated egg)	Original	24·0	2·48		5·81	
	1st top portion	7·5*	2·14	89	4·60	56
	1st lower portion	15·5	2·54		5·74	
	2nd top portion	1·2	1·94	111	3·54	112
	2nd lower portion	5·3	2·24		4·74	
(iv) Clarified membrane suspension (chorioallantois of entire eggs)	Original	85·0	2·60		7·57	
	1st top portion	17·8*	2·54	98	5·85	107
	1st lower portion	67·2	2·60		7·69	
	2nd top portion	1·2	2·30	70	5·23	95
	2nd lower portion	14·8	2·40		5·84	

In the four experiments shown in Table 3, it is clear that a considerable degree of separation of c.f.a. and infective virus was achieved. The concentration of infective virus in the top 1·2 ml. after the second spin was in each case reduced to about 0·5 p.c. of that in the original material (0·34, 0·50, 0·54 and 0·46 p.c.). In contrast, the concentration of c.f.a. was reduced by less than two-fold (to 70, 63, 29 and 50 p.c.). It may be noted that with materials derived from membranes, a greater portion of c.f.a. was sedimented with infective virus than was the case with the amniotic fluids. Whilst the recoveries of both components were within the experimental errors of the tests, it is likely that small losses occurred during the experiments because the mean recoveries of three of the experiments were 89 p.c. for infective virus and 95 p.c. for c.f.a.

It may be inferred from these experiments that because 99 p.c. of the infective virus was sedimented, at least 63 to 70 p.c. of the c.f.a. exists as a soluble entity in amniotic fluids and at least 29 to 50 p.c. in the suspensions made from membranes.

Washing infective virus.

An attempt was made to free virus from c.f.a. using clarified membrane suspensions from the chorioallantois of whole eggs after 48 hours' incubation. This material was spun at 10,000 *g.* for one hour and, after removal of the supernatant, the deposit was taken up in nutrient broth. The resuspended deposit was then spun again at 10,000 *g.* for one hour. The supernatant was removed and the deposit taken up in a small volume of broth. The samples were then titrated for c.f.a. and infective virus.

The results, given in Table 4 (i), show that the recovery of infective virus was good, suggesting that the techniques of separation and resuspension were adequate at least for the infective particle. Whilst the virus was concentrated 68-fold, in the final suspension, the c.f.a. was only concentrated 5-fold. Thus 7·3 p.c. of the c.f.a. appeared to be associated with infective virus. The recovery of the c.f.a. was 63 p.c. which, if correct, would mean that 12 p.c. of the c.f.a. had not been washed away from the virus.

It was suspected that the small amount of c.f.a. apparently associated with infective virus might be loosely attached. The experiment was therefore repeated in the same fashion but washing the deposit four times with broth. The conditions were as follows: Initial spin at 10,000 *g.* for 60 minutes. The first three washes using 45 ml. broth each time were each followed by a spin at 10,000 *g.* for 60 minutes, and the fourth wash, using 7 ml. broth, was followed by a spin at 12,000 *g.* for 50 minutes. Because the washings would be expected to contain relatively insignificant amounts of c.f.a. and infective virus, these were not titrated.

TABLE 4.

Showing the separation of c.f.a. from infective virus by washing the deposits obtained after successive spins at 10,000 g. for one hour. All values are given as $\log_{10}$ units.

Material	Sample	Volume (ml.)	c.f.u.	Recovery (p.c.)	i.u.	Recovery (p.c.)
(1) Clarified membrane suspension (chorio-allantois of entire eggs)	Original	98	2·60		7·65	
	1st supernatant	97	2·30		5·56	
	2nd supernatant	45	<1·70	63	5·63	104
	2nd deposit	1·5	3·30		9·53	
(ii) Clarified membrane suspension (chorio-allantois of entire eggs)	Original	96	2·27		8·08	
	1st supernatant	95	2·02	62	5·96	104
	4th deposit	1·73	2·83		9·84	
(iii) Amniotic fluid	Original	98	2·07		9·10	
	1st supernatant	97	1·98	83	7·80	120
	4th deposit	1·0	2·52		11·16	

The results (Table 4, ii) were strikingly similar to those in the previous experiment. The virus was concentrated 57·5 times with no evident loss, and the c.f.a. 3·6 times. This suggests that 6·3 p.c. c.f.a. is associated with infective virus or, taking into account the loss of c.f.a., 10·6 p.c.

The extra washes had but little effect on the c.f.a. which was associated with infective virus. Three possible reasons may be suggested for this residual activity:

(1) The existence of "particulate" c.f.a. of a size commensurate with that of infective virus. This includes the possibility of infective virus behaving as complement fixing antigen.

(2) The adsorption of soluble c.f.a. to virus particles.

(3) The adsorption of soluble c.f.a. to particles of similar size to the virus.

It was thought that the third possibility might be eliminated by washing the virus derived from embryonic fluids. This would not be expected to contain as much cellular debris as material derived from membranes. Thus, if the residual activity were due entirely to adsorption of soluble antigen to particles of host tissue, little or no c.f.a. should be sedimented.

The experiment was carried out as described above, but using virus derived from amniotic fluid which had been obtained by Nagler's (1946) method. After clarification by spinning at 2,400 *g.* for 20 minutes, the virus was repeatedly washed as in the previous experiment.

The results shown in Table 4 (iii), show that whilst the infective virus was concentrated 115 times, the c.f.a. was only concentrated 2·8 times in the final material. The recoveries of both c.f.a. and infective virus were good. Thus it may be inferred that 2·5 p.c. c.f.a. (or taking into account the losses which occurred, 3·6 p.c. c.f.a.) was sedimented with the virus. Whilst the separation was not complete, significantly less c.f.a. was associated with virus in amniotic fluid than was the case with clarified membrane suspensions.

Hence, from the results obtained from the two types of experiment (sedimentation of infective virus and washing infective virus) up to 90 p.c. of the c.f.a. behaves as a soluble antigen which is separable from the elementary body. In particular, the complement fixation test employed here detects a soluble antigen rather than a particulate entity and may be used to follow the formation of soluble antigen in the growth cycle.

An unsuccessful attempt to demonstrate the existence of two serologically distinct complement fixing antigens.

Brown (1953) has shown that the soluble c.f.a. of herpes virus is serologically distinct from the particulate antigen which reacts with neutralizing antibody. It was thought desirable to ascertain whether the c.f.a. which had been found above to sediment with infective virus was serologically distinct from the soluble component.

An attempt was made to absorb neutralizing antibody from immune serum while leaving the antibody which reacts with the soluble antigen. The serum was mixed with an equal volume of washed concentrated virus, shaken well and held at 4° C. for 18 hours. It was found that the infectivity of the mixture had almost completely disappeared by this time, while the treated serum still neutralized 100 i.u. by the method of Burnet and Lush (1939).

Clearly, even after treatment with washed concentrated virus ($1·5\times10^{11}$ i.u. per ml.), free neutralizing antibody remained in the serum. Whilst it might be possible to absorb out neutralizing antibody using a higher virus/serum ratio, this was not attempted because the further concentration of virus would have been cumbersome and costly and the dilution of serum would have interfered with the tests for residual antibodies after absorption.

Time appearance of c.f.a. and infective virus in the growth cycle.

The observation of Hoyle (1948), and by Henle and Henle (1949), that the soluble antigen of influenza virus can be detected before any other manifestation of viral activity occurs has led to the suggestion that the soluble antigen may be virus precursor. Fulton (1949) has brought evidence to show that the soluble and particulate antigens of this virus appear simultaneously. With herpes virus, Scott, Coriell, Blank and Gray (1953) were unable to detect an interval between the appearance of infective virus and c.f.a. Their results emphasize the fact noted here that c.f.a. can only be detected when high titres of infective virus are present. This makes it difficult to study the temporal relationship of the appearance of c.f.a. in the first intracellular growth cycle.

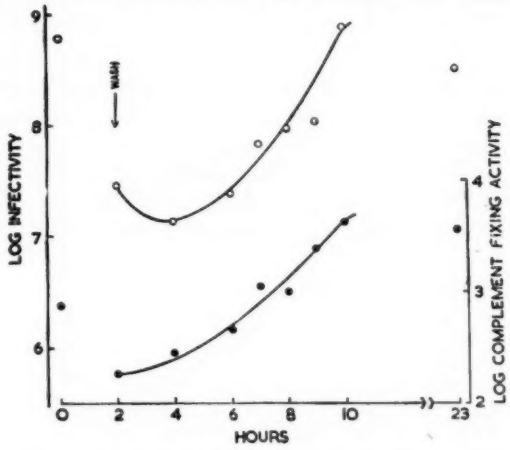

Fig. 1. Showing the increases of c.f.a. ($\log_{10}$ c.f.u.) and infective virus ($\log_{10}$ i.u.) in de-embryonated preparations which had each been inoculated with $\log_{10}$ 8·8 herpes virus.

○ Infective virus.
● Complement fixing antigen.

In the present investigation, this difficulty has been overcome by using the de-embryonated egg with a very large inoculum. In this way it was found possible to detect c.f.a. within 2 hours of inoculation. A number of de-embryonated eggs were inoculated with pooled amniotic fluids which contained approximately 10^9 i.u. per ml. The inoculum had been prepared by Nagler's (1946) method. Five ml. amounts were allowed to revolve in each egg for two hours. The eggs were then washed out thrice with physiological saline and into each was put 5·0 ml. glucose-antibiotic-Ringer's solution. At intervals the eggs were harvested two at a time and, after washing, the membranes were homogenized and titrated for c.f.a. and infective virus.

The results given in Fig. 1 are those of one of three experiments made as described above. The curves have been drawn taking into consideration the results of all the experiments, each of which showed the following points: (i) The content of infective virus increased steadily from the 4th hour to reach a maximum at the 10th hour. (ii) By the 22nd to 24th hours the content of infec-

tive virus was always less than at the 10th hour (in one case it had fallen by 1·7 $\log_{10}$). (iii) The c.f.a. content also rose to reach a maximum at the 10th hour. Titrations at the 2nd hour were made in two of the experiments, but only in the case shown (Fig. 1) was c.f.a. detected at this time.

The interpretation of the results is difficult because it is not possible to be certain at what time the content of infective virus begins to rise.

Firstly, taking into account the accuracy of the method of titrating infective virus, there is no indication that any increase occurs before the 6th hour. However, in all three experiments the infectivity titre increased between the 4th and 6th hours. Moreover, although in the previous paper (Wildy, 1954) it was found that infective virus did not increase until after the 6th hour, this was when using an inoculum of —1/1,000 of the amount; Scott *et al.* (1953) have shown that the duration of the eclipse phase of herpes virus is inversely proportional to the size of the inoculum.

Secondly, assuming that the curves faithfully represent the changes which occurred in the membranes, there is a clear indication that new infective virus was being formed between the 4th and 6th hours, but it cannot be stated that no new virus was formed between the 2nd and 4th hours, because at this time the picture was obscured by virus attached to the surfaces of the membranes and not multiplying. Attempts had been made to remove such virus by use of neutralizing serum, but this resulted in the membranes harvested at the 2nd hour becoming anticomplementary. Thus no advantage was gained.

DISCUSSION.

The results of the ultracentrifuge studies confirm the findings of Hayward (1949), Dudgeon (1950) and of Brown (1953) that the c.f.a. of herpes simplex virus is largely separable from the infective particle. The contrary finding of Fowler (1950) cannot be explained on the basis of the results obtained here because the conditions of testing used by him were very different. He used a short period for fixation (one hour at 37° C.) and used a guinea-pig serum as a source of antibody. Because we found that up to 90 p.c. of c.f.a. was separable from infective virus, the complement fixation test used here can be regarded as a measure of soluble antigen.

Although most of the c.f.a. was separable from infective virus, in all the preparations tested here it was never found possible to obtain infective virus devoid of complement fixing activity. This residual activity might be caused by particulate c.f.a., which has been demonstrated in suspensions of herpes virus by Brown (1953). On the other hand, soluble antigen may be associated with elementary bodies or particles of similar size derived from the host cells. It is believed that this is, in fact, the case because with virus derived from membranes significantly more c.f.a. was sedimented than with virus in amniotic fluid.

It is probable that the soluble c.f.a. of herpes virus is an entirely intracellular constituent whose formation is necessary for the production of new virus, but whose release is not. This is suggested by the following two facts: (a) soluble c.f.a. has constantly been found associated with *growing* virus, and (b) c.f.a. was largely released into the embryonic fluids after death of the embryos.

The rôle played by soluble antigens in the virus-host cell relationship is enigmatic. They have variously been considered as virus precursors, building blocks, metabolic products or products of disintegration (Hoyle, 1948, Jawetz *et al.*, 1951, and Brown, 1953). Fulton has suggested that soluble antigen of influenza virus may represent a matrix substance in which new virus is formed.

Our findings give no clear cut indications as to whether any of these suggestions are correct.

No evidence was obtained to support the suggestion that c.f.a. forms an integral part of infective virus. It had been hoped that the question of whether c.f.a. precedes new infective virus in the growth cycle might have been answered and thus have at least established whether the soluble antigen is a virus precursor. The conditions employed in following the temporal relationship of the formation of c.f.a. and infective virus were such that c.f.a. could readily be detected in the first intracellular growth cycle, and it is believed that no technical improvement may be expected from this type of experiment with the methods of titration at present available. It is obvious that the periods in which new infective virus and c.f.a. are formed largely coincide, but, for the reasons previously given, the results do not provide unequivocal evidence of the relative times at which these components first appear.

Taken as a whole, the results obtained here may be reconciled with the hypothesis that soluble complement-fixing antigen represents a matrix substance or scaffolding on which viral components are synthesized and assembled. Ackermann and Kurtz (1952) have suggested that mitochondria may be directly concerned in the growth of herpes simplex virus, and it is possible that the matrix substance (c.f.a.) is laid down on these structures from which it can only be recovered by some disruptive process such as autolysis. Moreover, the small portion of c.f.a. which was always found to sediment with infective virus may well represent matrix material which is still attached to mitochondrial fragments.

SUMMARY.

The complement-fixing antigen of herpes simplex virus was consistently detectable in preparations which had a high content of infective virus. The findings that most of the c.f.a. exists as a soluble entity has been confirmed. It has been shown that up to 90 p.c. of the complement fixing activity of a virus preparation is due to the soluble component.

Under suitable conditions, it was possible to demonstrate the formation of c.f.a. in the first intracellular growth cycle. It has been shown that this mostly occurs at the same time as the formation of new infective virus, but it was not found possible to obtain unequivocal evidence of the relative times at which the c.f.a. and infective virus first appeared.

Complement fixing antigen is essentially an intracellular component which, it is believed, may represent a matrix or scaffolding necessary for the multiplication of infective virus.

Acknowledgment. It is a pleasure to thank Sir Macfarlane Burnet for his interest and advice.

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THE GROWTH CURVE OF VACCINIA VIRUS ON THE CHORIOALLANTOIS

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(Accepted for publication 30th March, 1954.)

The virus of influenza, growing in the chick embryo allantoic cavity, was demonstrated to undergo an "eclipse phase" by Henle, Henle and Rosenberg in 1947. Other laboratories confirmed this finding, and recently Scott *et al.* (1953) and Wildy (1954) have demonstrated the same phenomenon with herpes simplex virus on the chorioallantoic membrane. Briody and Stannard (1951) determined the growth curve of vaccinia virus on the chorioallantois; using a relatively large inoculum, they found that by the end of one hour the amount of viable virus on the membrane had greatly decreased. The virus reappeared in two steps at 8 and 16 hours, respectively, and increased thereafter. These findings indicated the existence of an eclipse phase; but it is to be noted that the authors employed sand and mortar and pestle to mince the membranes, a procedure which may itself destroy virus. Crawford and Sanders (1952) noted a comparable decline in infectivity when vaccinia virus was inoculated onto tissue cultures of rabbit skin. After 10 hours increasing amounts of new virus appeared.

The present work was undertaken to gather further evidence as to whether there was an eclipse phase during the multiplication of vaccinia virus on the chorioallantois.

One of the earliest difficulties encountered in our study was the finding that grinding techniques currently used in these laboratories were destructive to various viruses, including vaccinia. An alternative shearing, or extrusion method of disrupting cells, has been used in this work.

Materials and Methods.

Vaccinia virus. Strain Gillard was used in the form of a suspension of infected chorioallantoic membrane. The virus was inoculated onto the chorioallantoic membranes of chick embryos previously incubated at 39° C. for 11 days. Incubation was continued at 37° C. for 48 hours, by which time a confluent growth of pocks was evident. The membranes were removed from the eggs and ground in a porcelain mortar with alundum. One ml. of beef heart infusion broth was added per membrane and the grinding repeated. Centrifugation for 5 minutes in an angle centrifuge at 4,000 r.p.m. yielded a clear supernatant. An equal volume

of pure glycerol was well mixed with the supernatant. The glycerolated suspension of vaccinia virus was maintained at −20° C. It had a titre of 2×10^7 pock-producing particles per ml. and maintained this titre over a period of at least four months.

Influenza virus. Strain MEL was grown in the allantoic cavity of 10-day chick embryos. Virus was used in the form of freshly harvested allantoic fluid.

Herpes virus. Strain HF was used, which by many passages had become adapted to the chorioallantoic membrane.

Abrasives.

Alundum was obtained as a commercial abrasive. It was used either untreated or after the following washing procedure—20 gm. alundum were rinsed several times in concentrated hydrochloric acid until the supernatant was clear. The alundum was then boiled for 30 minutes in concentrated HCl and washed thoroughly in six rinses of distilled water. It was then boiled in distilled water for five minutes and rinsed six times. This last procedure was repeated and the alundum was then dried in an evaporating basin.

Powdered glass was made by grinding soda glass tubing to a powder, using a glass mortar and pestle. The powder was washed in boiling water, then five changes of glass distilled water, then dried in a glass petri dish.

Virus in or on inoculated membranes was titrated as follows: In the case of vaccinia virus and herpes simplex virus, inoculated chorioallantoic membranes were recovered from eggs by opening the shell over the artificial air space, cutting the membrane with scissors a little peripheral to the edge of the "dropped" portion of the membrane, and lifting the freed portion of membrane with forceps. The membrane was then ground by one of several methods as described later, and the resultant mash evenly suspended in beef heart infusion broth. It was centrifuged at 4,000 r.p.m. for five minutes. Tenfold dilutions of the supernatant were made in broth, and each dilution was placed in amounts of 0·05 ml. onto the chorioallantoic membranes of at least 6 eggs 11 days old. The latter membranes were harvested 48 hours later and pocks were counted.

In the case of MEL influenza virus, infectivity titrations were performed by the inoculation of virus dilutions into the allantoic cavity of 10-day chick embryos. Six eggs were used for each tenfold dilution of virus. Haemagglutinin was determined in allantoic fluid two days later.

Titration of haemagglutinin of MEL followed the pattern test techniques in general use in these laboratories.

EXPERIMENTAL.

An assessment of grinding techniques.

The construction of a growth curve of vaccinia virus depends on the estimation of virus present in or on the chorioallantoic membrane at various intervals after inoculation of a known amount of virus. This demands the emulsification of the membrane in a saline solution in such a way that all cells are disrupted, and all virus remains in the supernatant after centrifugation.

It soon became clear that the method generally used in these laboratories to emulsify tissue—grinding in a mortar with a pestle, using alundum as abrasive —was itself responsible for a considerable decrease in titre of viable vaccinia virus. Various abrasives were then tried, but all were unsatisfactory. Finally,

Mr. M. Simpson, of this Institute, designed a metal mincer[1] which proved satisfactory for the preparation of emulsions of infected membranes.

Two experiments illustrating these points are described. An appropriate dilution was made of vaccinia virus in beef heart infusion broth, and the dilution held at 20° C. during the course of the experiment.

A volume of 0·05 ml. of the same dilution was ground for one minute in a porcelain mortar, with 0·5 ml. of powdered alundum, using a porcelain pestle. One ml. of beef heart infusion broth was added and the grinding continued for 30 seconds. Centrifugation gave a clear supernatant which was titrated for viable virus.

In a similar fashion, virus was ground with alundum in a glass mortar with a glass pestle, in a glass mortar without abrasive, and in a porcelain mortar without abrasive. The resulting fluids were titrated at the end of these manipulations; the original untreated virus dilution was titrated at the same time.

TABLE 1.

Alteration of titre of viable vaccinia virus after grinding with abrasives or in porcelain mortars.

Conditions of grinding or treatment	Titre of viable virus in suspension, as pocks per ml. Experiment No. 1	Experiment No. 2
No grinding—control	2,000	260×10^4
Extruded through metal mincer	1,900	190×10^4
Ground in glass mortar, no abrasive	700	
Shaken with powdered glass, no grinding	280	
Ground in porcelain mortar, no abrasive	80	
Ground in glass mortar, alundum as abrasive	60	
Ground in porcelain mortar, alundum as abrasive	20	12×10^4
Ground in porcelain mortar, washed alundum as abrasive		6×10^4

The findings with vaccinia virus subjected to these procedures are given in Table 1. It is clear that under these circumstances, contact with either alundum, powdered glass or porcelain proved disastrous to the titre of viable virus in the suspension. A similar result was obtained by the use of alundum or powdered glass, which had previously been washed as described above.

Vaccinia virus suspension forced through the mincer, described above, retained its original titre.

[1]The barrel of stainless steel was of internal diameter 9·5 mm. and had a closely fitting smooth brass plunger. The clearance between plunger and barrel was approximately ·0075 mm. The machine was designed to hold 1 ml. below the plunger, and to have above the plunger an annular reservoir of volume 1·5 ml. The chorioallantoic membrane to be ground was placed in the barrel. A vice in the vertical position was used to force the plunger into the barrel so that the membrane was extruded into the upper reservoir, a process which caused disruption of all tissue cells and erythrocytes, as determined by microscopic examination.

The above procedures with alundum were repeated, one 12-day chorioallantoic membrane being added with each 0·5 ml. of abrasive. The same degree of reduction of titre of vaccinia was demonstrated. That is, the presence of a tissue emulsion, added separately from the vaccinia virus, did not affect the reduction in titre due to the use of abrasive. In parallel with this, a mixture of vaccinia virus suspension, and one chorioallantoic membrane, forced through the metal mincer did not show a significant drop in titre of virus, the figures being 100 pocks per ml. of original dilution and 68 pocks per ml. after mincing. (These figures are adjusted to allow for the dilution of the virus, which occurred when it was minced with the chorioallantoic membrane.)

A further experiment determined the effect of alundum in a porcelain mortar on the recovery of vaccinia virus from an infected membrane. A membrane showing vaccinia pocks was harvested from an egg, 14 days old, 48 hours after inoculation. The membrane was cut into two equal parts. The first part was ground with alundum and suspended in 1 ml. of nutrient broth. The virus in the supernatant was at a titre of 5×10^7 per ml. The second half was ground in the metal mincer described above, and similarly suspended in 1 ml. of broth. The titre of virus in the supernatant was $2\cdot5\times10^7$ per ml. It is apparent that grinding with alundum had not diminished the amount of vaccinia virus recoverable from the infected membrane which had been harvested 48 hours after inoculation.

MEL influenza virus. A volume of 0·05 ml. of allantoic fluid MEL virus was ground in a porcelain mortar with 0·5 ml of alundum, using a procedure as described for vaccinia virus. After the addition of 1 ml. of broth, the ID_{50} of virus for allantoic eggs was $10^{-0.6}$. The original allantoic fluid, similarly diluted, showed a titre of $10^{-4.4}$. Thus the alundum grinding had diminished the infectivity titre by a factor of $10^{3.8}$. A corresponding titration of haemagglutinin in other samples of control and treated MEL virus demonstrated a decrease in titre from 1:800 to 1:20, that is by a factor of $10^{1.6}$. The titre of MEL virus infectivity or haemagglutinin was unaffected by passage through the metal grinder.

However, MEL virus present in chick embryo lungs harvested from 16-day old embryos, was apparently not diminished in titre by grinding with alundum. Virus haemagglutinin was present to the same titre in suspensions of lungs prepared by alundum grinding, or by grinding in the metal mincer described above. MEL influenza virus resembles vaccinia in that the infectivity and haemagglutinin are diminished in amount when the virus suspension is ground with alundum; but when virus infected tissue is ground the virus titre apparently is not decreased.

Herpes simplex virus. Findings, essentially similar to those just described for vaccinia, have been made by Dr. P. Wildy in these laboratories in the case of herpes simplex virus (Wildy, 1954) (personal communication).

Technique adopted.

As a result of these observations, we have used the metal mincer described above in the determinations of the growth curve of vaccinia virus. From time to time during the work, a histological check was made of the disruption of all the cells of the tissue being minced.

Growth curve of vaccinia virus.

A series of 11-day embryos was inoculated onto the chorioallantois, each egg receiving 0·05 ml. of a suspension of vaccinia virus and then being incubated at 37° C. At various intervals thereafter the whole inoculated surface was removed from the egg and ground in the metal mincer. The amount of viable virus in the supernatant was estimated (Table 2). Up to the ninth hour there was a steady decrease in the amount of virus recoverable. At the tenth hour there was an increase in virus, and from then onwards the titre increased in almost logarithmic fashion.

TABLE 2.

Pock-producing particles of vaccinia virus recoverable from chorioallantoic membranes at various intervals after inoculation.

Time after inoculation	Pock-producing particles per membrane
Number inoculated	250
30 minutes	70
1 hour	350
2 hours	245
4 hours	155
7 hours	42
9 hours	18
10 hours	70
11 hours	95
12 hours	680
14 hours	1,600
16 hours	7,500
20 hours	47,000
24 hours	560,000

The essential features of this growth curve have been confirmed by three further experiments along similar lines.

We then wished to determine whether the initial decrease in titre was due to the existence of an eclipse phase. The possibilities had not been excluded, for example, (1) that the virus was multiplying in other tissues of the embryo and returning to the membrane from the tenth hour onwards, or (2) that there was a destruction by the chorionic cells of up to 90 p.c. of inoculated virus during the first nine hours after inoculation, and then a rapid series of binary fissions of the remaining viable virus particles from the ninth hour onwards. Our

technique was inspired by that described by Girardi *et al.* (1952), working with meningo-pneumonitis virus.

One embryo 12 days old was inoculated onto the chorioallantoic membrane with 200 pock-producing particles of vaccinia virus. The egg was incubated at 37° C. for 8 hours. The membrane was then removed from the egg and washed lightly by rinsing it in beef heart infusion broth for 5 seconds. The membrane was then divided into two equal halves. One half was ground by shearing stresses so that all cells were disrupted. The virus content of the mash was determined. No pock-producing particles were recovered. The other half of the original membrane was cut into 16 approximately equal portions, each of which was dropped whole onto the chorioallantoic membrane of a 12-day old embryo. The latter 16 eggs were incubated for 48 hours and the membranes harvested.

In 14 instances the tissue graft had survived; in two of these eggs there was one typical vaccinial pock on the host membrane immediately adjacent to the graft. In the other 12 eggs no vaccinial pock was present. In the 2 eggs in which the graft had not survived there was no viral pock. The 14 membranes bearing live transplants were each ground and passed to 6 further chorioallantoic eggs. In this manner each piece of grafted chorioallantois, together with the neighbouring host membrane, was demonstrated to have contained between 20 and 200 virus particles 48 hours after the transplant was performed.

This type of experiment was repeated on two occasions, each time with a result similar to that described above. We feel that this finding demonstrates an eclipse phase for vaccinia virus growing in the cells of the chorioallantoic membrane.

DISCUSSION.

We draw the following conclusions:

(1) Of the particles of viable vaccinia virus absorbed by the surface cells of the chorioallantoic membrane, the majority, if not all, enter an eclipse phase after absorption.

(2) In a population of infective vaccinia particles the entry into the eclipse phase occurs throughout a period of at least nine hours.

This slow "disappearance" of virus may be due to one or both of two factors. Either there may be a prolonged period during which absorption of the population to surface cells occurs or, more probably, there may be a considerable variation in the period elapsing between the instant of absorption of any one virus particle and the moment when that particle enters the eclipse phase.

(3) From the ninth hour onwards there is a steady liberation of virus, in a logarithmic fashion, from infected cells. Under the conditions of our work no stepwise increase in titre was noted. This lack may well have been due to

masking by the prolonged period during which initial entry of virus to cells occurred; or there may be no cell burst in the strict sense, but virus may be liberated in a continuous stream from each infected cell over a considerable period of time.

The use of a metal mincer, in place of the more usual grinding techniques with abrasive, has allowed the demonstration of this prolonged persistence of the viable virus in the inoculum after it has been placed on the membrane. The technique of mincing is particularly appropriate to the problem in hand, but the evidence suggests that the effect of abrasives is of no significance in the recovery of the virus from a membrane in which it has grown. Suspensions prepared as supernatants from ground membranes, or such virus particles placed on a fresh membrane, are rendered largely non-viable by contact with abrasives; but virus which is about to be, or has just been liberated from infected cells is not so affected by abrasives. These facts suggest that a change may occur in vaccinia or influenza virus particles soon after they are released from infected cells, but the available evidence does not allow an explanation of this phenomenon.

SUMMARY.

Grinding of vaccinia virus or influenza virus with alundum or powdered glass diminished the titre of viable virus.

For this reason a shearing or extrusion technique has been used to emulsify chorioallantoic membranes onto which vaccinia virus has been placed.

The amount of viable vaccinia virus recoverable from inoculated chorioallantoic membranes decreased slowly and steadily over a period of nine hours.

At and after ten hours there was a steady logarithmic increase in the amount of virus recoverable.

A modified tissue culture technique has been used to show an eclipse phase during the growth of vaccinia virus on the chorioallantoic membrane.

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THE DETERMINATION OF VITAMIN B_{12}-ACTIVITY IN THE ORGANS AND EXCRETA OF SHEEP

II. THE INFLUENCE OF COBALT ON THE PRODUCTION OF FACTORS POSSESSING VITAMIN B_{12}-ACTIVITY IN THE RUMEN CONTENTS OF SHEEP

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(Accepted for publication 5th April, 1954.)

The effect of the level of cobalt intake on the vitamin B_{12}-active material in the faeces of sheep has already been described (Dawbarn, Hine and Hughes, 1952). The present communication reports an examination of the effect of cobalt status on the vitamin B_{12}-activity in rumen contents.

METHODS.

Four microbiological assay methods were employed, each reported to be differently responsive to the various vitamin B_{12}-like factors which are present in rumen contents (Ford, 1953a, b). The procedures used in this laboratory for assay of vitamin B_{12} by *Lactobacillus leichmannii* tube assay and *Escherischia coli* mutant plate assay have already been discussed in detail (Dawbarn and Hine, 1954). An *E. coli* tube assay, described by Burkholder (1951), has been used with only minor modifications. In the fourth assay method the test organism was a chrysomonad, *Ochromonas malhamensis*,[1] which has been shown by Ford (1953b) to respond only to vitamin B_{12} itself (cyanocobalamin), and not to factors A, B and C or pseudovitamin B_{12}.

The estimates of vitamin B_{12}-activity made by the specific method of Ford (1953b) and the other three methods were correlated to indicate the presence of the various active substances in the rumen and their changing proportions under different experimental conditions.

The methods were first compared by assaying liver, since the vitamin B_{12}-activity in this organ is largely attributable to vitamin B_{12} itself. Estimates obtained by the four methods should therefore be substantially the same.

The standard extraction procedure adopted by us involved autoclaving the tissue for 15 minutes in an acetate buffer at pH 4·6 (extraction method A). Ford (1953b) reported that acetate has an inhibitory effect on the growth of *Ochromonas*, and recommended that extraction should be carried out by steaming for 30 minutes in water at pH 5 (extraction method B). We therefore compared the two procedures. It has already been shown (Dawbarn and Hine, 1954) that estimates of the vitamin B_{12} in liver made by the *E. coli* plate and *L. leichmannii* tube methods are the same. The results of the other experiments are reported in Table 1.

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[1]We are indebted to Mr. J. E. Ford, National Institute for Research in Dairying, Shinfield, Reading, for a culture of this organism.

TABLE 1.

The effect of the method of extraction and of the method of assay on the estimated vitamin B_{12} content of liver.

Expt. No.	Method of extraction*	Method of assay	No. of replicate samples	μg. B_{12}/gm. (wet wt.)
1	A	*L. leichmannii*	2	0·86
	B	*L. leichmannii*	2	0·87
2	A	*E. coli* tube	3	1·27
	A	*L. leichmannii*	3	1·13
	B	*E. coli* tube	3	1·26
	B	*L. leichmannii*	3	1·14
	B	*Ochromonas*	3	1·11
3	B	*E. coli* tube	2	1·25
	B	*L. leichmannii*	2	1·10
	B	*Ochromonas*	2	1·10

*A—15 minutes' autoclaving in acetate buffer at pH 4·6 (cyanide present).
B—30 minutes' steaming in water at pH 5·0 (cyanide present).

Statistical analysis showed that there was no significant difference between the results obtained by the two methods of extraction, and that the *L. leichmannii* and *Ochromonas* assays gave the same estimate for vitamin B_{12} in liver. A significantly greater estimate was obtained by the *E. coli* tube assay. This may perhaps be attributed to the presence of methionine, which can replace vitamin B_{12} for this organism. Alternatively, it may indicate the presence of traces of a factor more active for *E. coli* than for the other micro-organisms. Ford, Holdsworth and Porter (1953) found traces of factor A in cow and pig liver. The estimate of vitamin B_{12} by the *L. leichmannii* method includes any activity due to desoxyribosides.

The recovery of pure vitamin B_{12} added to liver before extraction was investigated (Table 2).

TABLE 2.

Recovery of pure vitamin B_{12} added to liver. Method of extraction B.

Method of assay	No. of replicate samples	B_{12} added μg./gm.	B_{12} found μg./gm.	5 p.c. fiducial limits	Sigce. of deviation from quantitative recovery
E. coli tube	2	0	1·25	1·235–1·265	
	2	1·00	2·28	2·265–2·295	$P < 0.05$
L. leichmannii	3	0	1·11	1·02 –1·20	
	2	1·00	2·09	1·98 –2·20	n.s.
Ochromonas	2	0	1·10	1·05 –1·15	
	2	1·00	2·12	2·07 –2·17	n.s.

Similar tests were made on rumen contents to determine whether the two extraction procedures were equally effective and to establish the quantitative recovery of added vitamin B_{12} for all assay methods. The results are presented in Table 3.

TABLE 3.

Recovery of pure vitamin B_{12} added to rumen contents.

Method of extraction	No. of replicate samples	B_{12} added µg./gm.	B_{12} found µg./gm.	*E. coli* tube assay: 5 p.c. fiducial limits	*E. coli* tube assay: Sigce. of deviation from quantitative recovery	*L. leichmannii* assay: B_{12} found µg./gm.	*L. leichmannii* assay: 5 p.c. fiducial limits	*L. leichmannii* assay: Sigce. of deviation from quantitative recovery	*Ochromonas* assay: B_{12} found µg./gm.	*Ochromonas* assay: 5 p.c. fiducial limits	*Ochromonas* assay: Sigce. of deviation from quantitative recovery
A	2	0	0·46	0·45–0·48		0·43	0·38–0·48				
	2	0·66	1·10	1·08–1·11	$P < ·05$	1·05	1·01–1·10	n.s.			
B	2	0	0·49	0·48–0·51		0·46	0·41–0·51		0·37	0·35–0·39	
	2	0·69	1·19	1·18–1·21	n.s.	1·13	1·09–1·18	n.s.	1·05	1·03–1·08	n.s.

It is apparent that the estimated vitamin B_{12} was greater when extraction was carried out by method B. The difference was statistically significant ($P < \cdot 001$) for the *E. coli* tube method but not for the *L. leichmannii* method.

We have examined the rumen contents of sheep collected under the following conditions.

Sheep with rumen fistulae.

Rumen contents were collected under the direction of Mr. H. R. Marston from some of his experimental sheep in which rumen fistulae had been established. These animals were confined in metabolism cages and were subsisting on a cobalt-deficient diet of wheaten-hay chaff containing 5 p.c. gluten (Marston, H. R.). Repeated samples of rumen contents were withdrawn from the animals which were treated as follows:

(1) To two sheep, Nos. 9029 and 9047, cobalt was administered, 1 mg. Co *per os* per day for 14 days, and then withheld. Samples of rumen contents were obtained 2 hours and 24 hours after the last dose of cobalt, and thereafter at intervals for 133 days (Tables 4 and 5).

(2) Cobalt was again administered to one of the above animals (No. 9029) but the diet, instead of being fed *ad lib.*, was restricted to the amount consumed by a cobalt-deficient sheep, No. 9151. Samples of rumen contents were obtained from these pair-fed sheep until the food intake dropped to 1 gm. per day (Tables 6 and 7).

(3) Sheep No. 9029, after the period of restricted food intake in (2), was fed *ad lib.* for one month while the administration of cobalt was continued. Cobalt was then withheld and samples of rumen contents were collected at intervals from 2 hours to 14 days thereafter (Table 8).

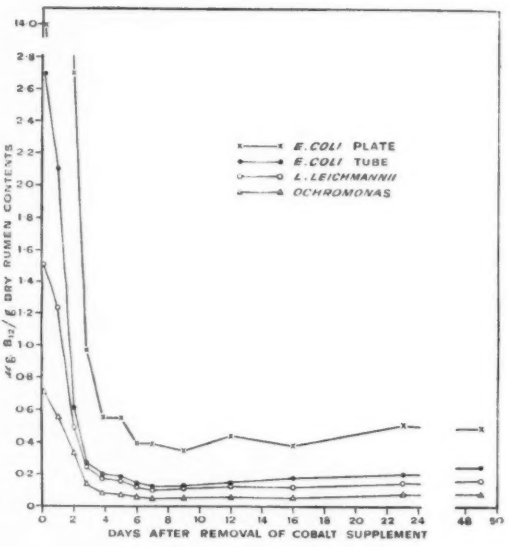

Fig. 1. The effect of the withdrawal of supplementary cobalt on the vitamin B_{12}-activity of rumen contents (Table 4).

Intact Sheep.

(1) An intact sheep (No. 1109) was fed on the wheaten-hay chaff diet, and 1 mg. Co *per os* per day was given for 15 days. The animal was then slaughtered and the contents of the rumen, abomasum, the whole of the small intestine and the distal end of the caecum were collected and representative samples were assayed (Table 9).

(2) Four sheep off pasture were slaughtered, the rumen contents were collected and representative samples were assayed (Table 10).

TABLE 4.

Vitamin B_{12}-activity of rumen contents of sheep No. 9029. 1 mg. Co per os *per day for 14 days, then Co withheld.*
Diet: *Wheaten-hay chaff containing 5 p.c. gluten,* ad lib.

Sample No.	μg. B_{12}-activity per gm. dry rumen contents: *E. coli* plate, a	*E. coli* tube, b	*L. leichm.*, c	*Ochrom.*, d	Ratio a	b	c	d	p.c. dry wt.	Food intake gm. per day	Cobalt in fodder p.p.m.	Time after final administration of cobalt
A1	14·1	2·7	1·51	·71	20	3·9	2·1	1	5·1	1003	0·12	2 hours
2	10·0	2·1	1·23	·55	18	3·8	2·2	1	6·5	1012	„	24 „
3	2·7	·61	·49	·33	8·2	1·8	1·5	1	7·5	886	„	48 „
4	0·97	·27	·24	·137	7·1	2·0	1·7	1	9·3	994	„	68 „
5	0·55	·20	·170	·082	6·8	2·5	2·1	1	9·8	1020	„	92 „
6	0·55	·185	·154	·076	7·3	2·4	2·0	1	7·2	952	„	5 days
7	0·39	·147	·118	·057	6·8	2·6	2·1	1	10·3	770	„	6 „
8	0·39	·122	·100	·048	8·0	2·5	2·1	1	10·1	864	„	7 „
9	0·35	·126	·112	·053	6·7	2·4	2·1	1	9·6	961	„	9 „
10	0·44	·150	·122	·057	7·8	2·6	2·1	1	8·6	952	„	12 „
12	0·38	·189	·111	·054	7·0	3·5	2·1	1	9·3	965	„	16 „
14	0·51	·20	·147	·075	6·8	2·7	2·0	1	9·1	926	„	23 „
18	0·49	·25	·167	·077	6·3	3·2	2·2	1	8·4	898	„	49 „
21	0·51	·21	·159	·071	7·2	3·0	2·2	1	8·5	1028	„	91 „
22	0·49	·25	·145	·092	5·4	2·8	1·6	1	7·1	866	„	112 „
23	1·29	·66	·42	·142	9·1	4·7	3·0	1	6·2	844	·016	133 „

Rumen contents obtained through a fistula. Samples collected immediately prior to feeding except A1, A2, A3: 4 to 5 hours after feeding.

TABLE 5.

Vitamin B_{12}-activity of rumen contents of sheep No. 9047. 1 mg. Co per os *per day for 14 days, then Co withheld.*
Diet: *Wheaten-hay chaff containing 5 p.c. gluten,* ad lib.

Sample No.	μg. B_{12}-activity per gm. dry rumen contents: *E. coli* plate, a	*E. coli* tube, b	*L. leichm.*, c	*Ochrom.*, d	Ratio: a	b	c	d	p.c. dry wt.	Food intake gm. per day	Cobalt in fodder p.p.m.	Time after final administration of cobalt
B1	11·7	2·2	1·57	·84	14	2·6	1·9	1	8·1	875	·012	2 hours
2	12·1	2·3	1·57	·94	13	2·4	1·7	1	7·9	843	„	24 „
3	4·3	1·21	1·15	·75	5·7	1·6	1·5	1	7·5	884	„	48 „
4	1·65	·54	·47	·34	4·8	1·6	1·4	1	8·5	680	„	68 „
5	1·03	·43	·34	·187	5·5	2·3	1·8	1	6·8	496	„	92 „
6	·59	·22	·139	·074	8·0	2·9	1·9	1	7·4	520	„	5 days
7	·50	·165	·101	·068	7·4	2·4	1·5	1	7·8	657	„	6 „
8	·47	·171	·095	·070	6·8	2·4	1·4	1	7·6	685	„	7 „
9	·36	·153	·081	·067	5·4	2·3	1·2	1	9·4	819	„	9 „
10	·51	·163	·102	·083	6·1	2·0	1·2	1	9·5	898	„	12 „
22	·52	·190	·123	·086	6·1	2·2	1·4	1	8·3	987	„	112 „
23	·81	·29	·180	·100	8·1	2·9	1·8	1	7·8	758	·016	133 „

Rumen contents obtained through a fistula. Samples collected immediately prior to feeding except B1, B2, B3: 4 to 5 hours after feeding.

TABLE 6.

Vitamin B_{12}-activity of rumen contents of sheep No. 9029. 1 mg. Co per os *per day, after 135 days without Co.*
Diet: *Wheaten-hay chaff containing 5 p.c. gluten; pair fed with Co-deficient sheep No. 9151.*

Sample No.	μg. B_{12}-activity per gm. dry rumen contents				Ratio				p.c. dry wt.	Food intake gm. per day	Cobalt in fodder p.p.m.	Time after re-administration of cobalt
	E. coli plate, a	*E. coli* tube, b	*L. leichm.*, c	*Ochrom.*, d	a	b	c	d				
C3	5·7	·80	·82	·41	14	2·0	2·0	1	6·1	642	·012	3 days
4	8·2	1·64	1·49	·49	17	3·3	3·0	1	6·1	488	„	10 „
5	11·5	1·92	1·92	·70	16	2·7	2·7	1	6·0	442	„	17 „
6	8·2	1·29	1·39	·45	18	2·8	3·1	1	5·6	350	·039*	27 „
9	14·8	2·1	1·82	·80	18	2·6	2·2	1	7·7	370	„	63 „
10	5·4	1·38	1·23	·59	9·1	2·4	2·1	1	3·9	32	„	78 „
11	3·7	·97	1·00	·56	6·7	1·7	1·8	1	3·6	1	„	80 „

* New season's chaff. Rumen contents obtained through a fistula.

TABLE 7.

Vitamin B_{12}-activity of rumen contents of cobalt-deficient sheep No. 9151.
Diet: *Wheaten-hay chaff containing 5 p.c. gluten*, ad lib.

Sample No.	μg. B_{12}-activity per gm. dry rumen contents				Ratio				p.c. dry wt.	Food intake gm. per day	Cobalt in fodder p.p.m.	Time after final administration of cobalt
	E. coli plate, a	*E. coli* tube, b	*L. leichm.*, c	*Ochrom.*, d	a	b	c	d				
D3	·68	·29	·22	·108	6·3	2·7	2·1	1	8·5	482	·012	284 days
4	·43	·180	·146	·065	6·5	2·7	2·3	1	8·9	498	„	291 „
5	·81	·42	·34	·118	6·8	3·6	2·9	1	8·5	359	„	298 „
6	·97	·25	·24	·077	12·5	3·2	3·1	1	6·2	350	·039	307 „
9	·35	·21	·157	·049	7·3	4·3	3·2	1	9·9	584	„	342 „
10	·35	·155	·122	·030	11·9	5·1	4·0	1	5·1	32	„	355 „
11	too low to estimate	·065	·059	·021		3·1	2·8	1	2·9	1	„	357 „

Rumen contents obtained through a fistula.

TABLE 8.

Vitamin B_{12}-activity of rumen contents of sheep No. 9029. 1 mg. Co per os *per day for 4 months, then Co withheld.*
Diet: *Wheaten-hay chaff containing 5 p.c. gluten, 1050 gm. per day. Cobalt in fodder, ·037 p.p.m.*

Sample No.	μg. B_{12}-activity per gm. dry rumen contents				Ratio				p.c. dry wt.	Time after final administration of cobalt
	E. coli plate, a	*E. coli* tube, b	*L. leichm.*, c.	*Ochrom.*, d	a	b	c	d		
F1	8·9	3·2	1·89	0·56	16	5·7	3·4	1	6·0	2 hours
2	5·7	2·1	1·19	0·40	14	5·2	3·0	1	8·3	24 „
3	5·8	2·0	1·25	0·53	11	3·8	2·4	1	8·5	36 „
4	2·1	0·88	0·58	0·26	8·1	3·4	2·2	1	6·7	48 „
5	2·4	0·95	0·61	0·29	8·3	3·3	2·1	1	7·9	60 „
6	1·29	0·62	0·38	0·154	8·4	4·0	2·5	1	6·0	72 „
7	0·90	0·41	0·25	0·092	9·8	4·5	2·7	1	7·0	97 „
9	0·44	0·28	0·175	0·069	6·4	4·1	2·5	1	7·6	5 days 20 hours
10	0·69	0·37	0·26	0·069	10	5·3	3·8	1	5·7	7 days
11	0·59	0·31	0·181	0·050	12	6·2	3·6	1	5·6	10 „
13	0·54	0·34	0·22	0·075	7·2	4·5	2·9	1	6·0	14 „

Rumen contents obtained through a fistula—samples collected 4 to 6 hours after feeding except F3 and F5, 16 hours, and F9, 24 hours after feeding.

TABLE 9.

Vitamin B_{12}-activity of contents of the alimentary tract of sheep No. 1109. 1 mg. Co per os per day for 15 days prior to slaughter.

Diet: *Wheaten-hay chaff* ad lib.

Section of alimentary tract	μg. B_{12}-activity per gm. (dry wt.)				Ratio				p.c. dry wt.
	E. coli plate, a	*E. coli* tube, b	*L. leichmannii* c	*Ochromonas* d	a	b	c	d	
Rumen	12·2	3·1	2·3	1·16	10	2·6	2·0	1	12·4
Abomasum	9·6	2·8	2·1	·94	10	3·0	2·2	1	10·0
Small intestine	16·3	5·2	4·3	1·55	10	3·4	2·8	1	7·5
Caecum	11·2	1·87	1·07	·54	21	3·5	2·0	1	11·2

TABLE 10.

Vitamin B_{12}-activity of rumen contents of sheep off pasture.

Sheep No.	μg. B_{12}-activity per gm. dry rumen contents				Ratio				p.c. dry wt.	Date
	E. coli plate, a	*E. coli* tube, b	*L. leichm.* c	*Ochrom.* d	a	b	c	d		
9028 wether	13·2	5·8	5·5	4·4	3·0	1·3	1·2	1	8·4	12/8/53
W5/529 pregnant ewe	12·5	3·3	3·1	2·2	5·7	1·5	1·4	1	8·3	23/9/53
635 wether	9·0	2·5	2·4	1·42	6·3	1·8	1·7	1	5·9	24/9/53
	3·2	·98	·58	·49	6·5	2·0	1·2	1	10·7	22/10/53

RESULTS AND DISCUSSION.

The whole of the results of the assays have been incorporated in Tables 4–10. Certain conclusions are immediately apparent.

The withdrawal of supplementary cobalt from sheep subsisting on a cobalt-deficient ration caused a spectacular fall in the vitamin B_{12}-activity of the rumen contents. In seven days the activity as determined by the four assays, *E. coli* plate, *E. coli* tube, *L. leichmannii* and *Ochromonas*, fell (for sheep No. 9029) to 3, 4·5, 7 and 7 p.c. of the previous values, while the corresponding figures for sheep No. 9047 were 4, 8, 6 and 8 (Tables 4 and 5, Fig. 1). When No. 9029 was subjected to this treatment for a second time the activity fell in six days to 5, 9, 9 and 12 p.c. (Table 8). The greater part of this decline took place in the first three days. Provided that appetite was maintained there was no further loss of vitamin B_{12}-activity after the first week. In the case of sheep No. 9151, however, appetite failed after 49 weeks on the cobalt-deficient diet and a further fall in vitamin B_{12}-activity occurred (Table 7). It must be pointed out that the wheaten-hay chaff diet was not devoid of cobalt, but contained 0·01 to 0·04 parts per million.

In the presence of adequate cobalt the vitamin B_{12}-activity, as determined by the *E. coli* plate assay, was between 10 and 20 times the true vitamin B_{12} content, as measured by the *Ochromonas* assay. On withdrawal of cobalt the ratio fell to less than 10 and usually remained below 10 (Tables 4, 5, 7, 8). A similar effect of cobalt was observed when sheep faeces were assayed by the *E. coli* plate and *L. leichmannii* methods (Dawbarn, Hine and Hughes, 1952). When the concentration of vitamin B_{12}-activity, measured by the *E. coli* plate method, was greater than 5·5 μg. B_{12}/gm. dry weight, the ratio of this activity to true vitamin B_{12} was always 10 or greater than 10 provided the sheep were subsisting on the wheaten-hay chaff diet. However, different results were obtained with sheep off pasture (Table 10). The rumen contents of one of these animals, No. 9028, had a vitamin B_{12}-activity of 13·2 μg. B_{12}/gm. by the *E. coli* plate method, while the true vitamin B_{12} content was 4·4, a ratio of only 3 to 1. This may be due to the nature of the diet or, possibly, to the fact that the cobalt was ingested as an integral part of the fodder and not as a drench given once in 24 hours.

Hale, Pope, Phillips and Bohstedt (1950) and Hoekstra, Pope and Phillips (1952) measured the vitamin B_{12}-activity in the rumen contents of sheep, using respectively a chick assay and the *L. leichmannii* assay. They found a much greater concentration in the rumen contents of sheep receiving cobalt than of cobalt-deficient animals.

The experimental results presented by us furnish material for speculation on the identity and proportions of the various vitamin B_{12}-active factors in the rumen. Definite conclusions, however, must await a separation of the factors by ionophoresis. This work is now in progress.

SUMMARY.

The vitamin B_{12}-activity in the rumen contents of sheep was determined by four microbiological methods, viz., *E. coli* mutant plate assay and *E. coli* mutant, *L. leichmannii* and *Ochromonas* tube assays.

Rumen contents were collected from sheep with rumen fistulae and from intact sheep which were subjected to different experimental treatments.

The withdrawal of supplementary cobalt from sheep subsisting on a cobalt-deficient diet caused a marked fall in the vitamin B_{12}-activity of the rumen contents. The greater part of this decline took place in the first three days. Provided that appetite was maintained, there was no further loss of vitamin B_{12}-activity after the first week.

The true vitamin B_{12}, as measured by the *Ochromonas* assay, was always less than the activity estimated by any of the other methods. The *E. coli* plate assay invariably gave substantially higher results than the other methods. This was particularly marked in the case of sheep subsisting on a wheaten-hay chaff diet with supplementary cobalt.

Acknowledgments. We would like to thank Mr. H. R. Marston, F.R.S., Chief of the Division of Biochemistry and General Nutrition of the C.S.I.R.O., for advice and assistance, and for the suggestion that these measurements be made on his experimental animals. We are also indebted to Mrs. E. Bofinger for carrying out the statistical analyses referred to in the text, to Mr. R. M. Smith for the collection of the rumen samples and to Mr. A. Snoswell for the cobalt estimations.

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PROTECTION OF LABORATORY RABBITS AGAINST MYXOMATOSIS BY VACCINATION WITH FIBROMA VIRUS

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(Accepted for publication 17th February, 1954.)

Myxomatosis of rabbits was first recognized when outbreaks occurred in laboratory rabbits in South America. It was from one such outbreak that Sanarelli (1898) recovered the virus. Similar outbreaks have occurred in European rabbits (*Oryctolagus cuniculus*) bred for both laboratory and commercial use in various parts of South America (Aragao, 1927; Moses, 1940), and since 1930 in South California (Kessel, Prouty and Meyer, 1931; Vail and McKenney, 1943).

With the introduction and wide dissemination of myxoma virus through South-eastern Australia (Ratcliffe, Myers, Fennessy and Calaby, 1952) it was foreseen that similar outbreaks might occur in laboratory rabbits in Australia. The first such episode occurred in January, 1952, at the Baker Institute for Medical Research, in Melbourne, Victoria. A year later outbreaks occurred at two hospitals in Sydney, N.S.W. The occurrence of widespread epizootics of myxomatosis in France during the summer of 1953, and its spread to England later that year, have emphasized the necessity for a satisfactory vaccination procedure.

Shope's (1932) demonstration that fibroma and myxoma were immunologically related has been repeatedly confirmed, but reports of the use of vaccination with fibroma virus as a means of controlling myxomatosis have been, on the whole, unfavourable. McKenney and Shillinger (1935) had difficulty in maintaining a potent preparation of fibroma virus, and their results were hardly a fair test of the efficacy of vaccination. Shope (1938) showed that inoculation with a potent preparation of fibroma virus conferred a high degree of protection to rabbits exposed to myxomatosis six weeks later, when the fibromata had completely regressed, but he did not test rabbits at longer intervals than this. Moses (1939), reviewing attempts at vaccination of rabbits with fibroma or treated myxoma virus, concluded that there was no satisfactory method of vaccination and recommended "stamping-out" as the only way of eliminating the disease from rabbitries.

There is ample evidence in the literature (Hurst, 1937; Hyde, 1939) that resistance to myxomatosis arises very rapidly, probably within forty-eight hours

of the inoculation of fibroma virus, but there have been no satisfactory studies of the duration of the acquired immunity. The present report deals with the degree and duration of immunity to myxomatosis after vaccination with two different strains of fibroma virus.

Materials and Methods.

Myxoma virus.

The South American strain, strain B of Martin (1936), was used throughout. Its history has been briefly described by Bull and Mules (1944). Stock virus was prepared as described in the Appendix. Ampoules of the virus suspension were stored at −70° C., so that preparations of known virulence and titre were always available.

Fibroma virus.

Experiments were conducted with the OA strain and Boerlage strain, both kindly provided by Dr. Richard E. Shope. The OA strain was isolated by Shope in 1932 from the foot of a wild cottontail rabbit. The Boerlage strain was isolated by Shope in 1947 (Smith, 1952; Shope, 1953) from a rabbit captured by Mr. P. J. Boerlage in Jackson Township, Ocean County, New Jersey. The method of preparation of large amounts of high titre virus for vaccination is described in the Appendix.

Rabbits.

Laboratory rabbits (*Oryctolagus cuniculus*) were obtained from the several laboratories co-operating in this investigation, in particular the National University Animal Breeding Establishment, the Baker Institute for Medical Research, the Bacteriology School of the University of Melbourne, and the Walter and Eliza Hall Institute of Medical Research.

Titration.

Stock myxoma virus was titrated on the chorioallantois of the chick embryo. As shown previously (Fenner and Woodroofe, 1953), one pock on the chorioallantois was roughly equivalent to 10 ID_{50} for the rabbit.

Fibroma virus was titrated by the intradermal inoculation of rabbits. Several sites were inoculated with 0·1 ml. of serial tenfold dilutions, and the lesions observed seven days later. More precise titrations were sometimes carried out by using twofold dilutions of virus. The titre was calculated by the method of Reed and Muench (1938).

Complement-fixation test.

The myxoma and normal control antigens used in the complement-fixation tests were prepared from the chorioallantoic membranes of developing chick embryos (Lush, 1939), and the fibroma antigen consisted of the extract in calcium-magnesium saline of subcutaneous lesions, prepared as described in the Appendix.

The technique of the complement-fixation test has been described in some detail elsewhere (Fenner, Marshall and Woodroofe, 1953). Serial twofold dilutions of each sample of serum were tested for complement-fixing activity in the presence of three 50 p.c. haemolytic doses of complement and a standard dilution of antigen. The indicator system of sensitized red cells was added after fixation had taken place overnight at 4° C. The myxoma antigen consisted of a 1/8 dilution of infected egg membranes, and the fibroma antigen used was a 1/32 dilution of the extract of fibroma lesion material in calcium-magnesium saline.

Neutralization tests.

Tests for the neutralization of myxoma virus were carried out on the chorioallantoic membrane by the pock-counting technique (Lush, 1937). Undiluted serum, inactivated by heating at 56° C. for 30 minutes, was mixed with the appropriate dilutions of virus and held for 30 minutes at room temperature before inoculation of the eggs. The results obtained have been expressed as a percentage reduction in the count, compared with the normal serum control.

EXPERIMENTAL RESULTS.

The persistence of enhanced resistance after vaccination with OA fibroma virus.

The first experiments were carried out with the OA strain of fibroma virus. Adult rabbits were inoculated intradermally with about 1,000 ID_{50} of virus. Such doses usually produced a tumour which reached a maximum size of 15–20 mm. diameter about ten days after inoculation and then regressed. At monthly intervals after vaccination five or six animals were challenged with myxoma virus. In the first two groups the challenge was given as a titration involving several tenfold steps, six in the first instance and four in the second.

There was a rapid increase in the degree of resistance when the rabbits were challenged by the inoculation in several sites of large doses of myxoma virus. A month after vaccination all animals withstood the inoculation in six sites of a total dose of virus in excess of a million infectious doses. The local lesions at the sites of inoculation of the larger doses of virus appeared somewhat sooner than in the control rabbits. When well developed they were always clearly demarcated from the surrounding skin, and consisted of circular raised areas with a clearly defined edge. The reactions at all inoculation sites were quite large, reaching a diameter of 30 mm. or more. The centre was deep purple, surrounded by a pale pink zone and then by a thin haemorrhagic ring. Hurst (1938) has described the histological characters of these lesions, which exhibit many of the features of an allergic reaction. In all animals a few secondary skin lesions appeared, either on the flanks or on the ears. Unilateral conjunctivitis occurred in one animal. All animals recovered completely after mild attacks of myxomatosis. The titres of complement-fixing antibody to myxoma virus rose from about 1/40 before the challenge inoculation to the very high levels of 1/5,120 and 1/10,240 three weeks after challenge.

A month later, i.e., two months after vaccination, the response was quite different in spite of the fact that a much smaller total dose of virus was given. Four skin sites were inoculated with doses of about 10, 10^2, 10^3 and 10^4 ID_{50} of myxoma virus. Two animals died 15 and 18 days after inoculation, having suffered severe but modified attacks of myxomatosis. The remaining two rabbits were both very ill with a modified disease, but recovered. The titre of complement-fixing antibody to myxoma virus rose from 1/16 to the high levels of 1/1,024 and 1/2,048.

TABLE 1.

The clinical and serological responses in rabbits challenged with a single small dose of myxoma virus three and five months after vaccination with a single inoculation of about 1,000 ID_{50} of fibroma virus (OA strain).

Rabbit No.	Interval between vaccination and challenge inoculation	Dose of myxoma virus (ID_{50})	Clinical response: Local lesion	Clinical response: Secondary skin lesions	Clinical response: Blepharitis and conjunctivitis	Clinical response: Outcome	Serological response: Complement-fixation tests: Fibroma: before challenge	Fibroma: after challenge	Myxoma: before challenge	Myxoma: after challenge	Neutralization tests: Myxoma virus: before challenge	Myxoma virus: after challenge
											p.c.	p.c.
758	3 months	10	30,22,18*	few	moderate	moderately severe, recovery	2	64	64‡	2048	47§	98
761	3 months	10	44,40,23	many	slight	severe disease, recovery	2	64	4	1024	65	97
760	3 months	10	34,28,19	many	severe	severe disease, recovery	0	64	0	2048	87	99
762	3 months	10^3	37,34,23	few	nil	mild reaction, recovery	0	4	0	256	82	99
101	5 months	10	35,25,17	many	severe	†14 day||	0	—	16	—	8	—
102	5 months	10	30,27,20	many	severe	very severe disease, recovery	8	256	64	4096	90	98·5
103	5 months	10	33,27,23	many	slight	severe disease, recovery	0	32	16	256	74	98·8
104	5 months	10	30,27	many	severe	†14 day	4	—	0	—	84	—
105	5 months	10	31,24,15	many	mild	severe disease, recovery	0	16	8	1024	50	97·5

* 30, 22, 18 indicate diameters in millimetres, at time of maximum development, of the outer erythematous thickened zone, the pale thickened ring, and the central dark purple or necrotic area.
‡ reciprocals of serum dilution showing 50 p.c. haemolysis in complement-fixation test.
§ percentage reduction in pock count on chorioallantoic membrane of chick embryo.
|| † 14 day, etc., died of myxomatosis 14 days after challenge infection.

In both these tests the dose of virus used for infection of the vaccinated rabbits was considerably greater than would occur in naturally-transmitted disease. In testing rabbits three and five months after vaccination only one inoculation was made, containing either about 10 or 10^3 ID_{50}. The results are shown in Table 1, in which are set out both the clinical and serological responses.

It is apparent that, although the reaction of vaccinated rabbits to infection with myxoma virus differs from that of normal animals, the protection evident five months after the inoculation of the fibroma virus is slight. After the inoculation of only 10 ID_{50} of virus, two rabbits died and the other three suffered severe attacks of myxomatosis before their ultimate recovery.

The use of OA fibroma virus during an outbreak of myxomatosis in laboratory rabbits.

In January, 1952, myxomatosis was diagnosed in the rabbit-breeding colony of the Baker Institute for Medical Research, Melbourne. Eighteen out of thirty-four adult rabbits in one run, obviously secondary cases infected from an unrecognized case which had been killed two weeks earlier, were found to be in the same advanced stage of illness. All the sick rabbits and their immediate contacts were destroyed, and a week later all rabbits, except kittens less than one-month old, were vaccinated by the intradermal inoculation of about 100 ID_{50} of OA fibroma virus. The subsequent history of the outbreak need not be related in detail; suffice it to say that what threatened to be a disastrous outbreak of myxomatosis in a rabbit-breeding colony was terminated within a month of recognition by a combination of the slaughter of diagnosed cases and the vaccination of all other rabbits (except those less than one month old) with OA fibroma virus. Enough cases occurred in unvaccinated kittens to indicate that vaccination had played a significant part in curbing the epizootic.

Although vaccination with OA strain of fibroma virus was shown to be a useful measure in the presence of an outbreak of myxomatosis in laboratory rabbits, it did not appear to be sufficiently durable to be used as a routine prophylactic measure in rabbit breeding establishments. Experiments were therefore carried out to determine whether inoculation with fibroma virus followed one or two weeks later by the inoculation of myxoma virus would secure a more durable immunity without disturbing the health of the rabbits. Six young adult rabbits were inoculated, one and two weeks after vaccination, with either 10 or 10^3 ID_{50} of myxoma virus. The results are recorded in Table 2. Resistance to a dose of 10 ID_{50} was complete a week after the fibroma inoculation, but after two weeks there was a definite local response even to this small dose. There were transient local reactions after the inoculation of 10^3 ID_{50} one week after vaccination, and significant rises in the titre of myxoma neutralizing antibody. After a further week's interval there were definite reactions 2–3 cm. in diameter

TABLE 2.

The clinical and serological responses in rabbits challenged with a single small dose of myxoma virus one and two weeks after vaccination with a single inoculation of 1,000 ID_{50} of fibroma virus (OA strain).

Rabbit No.	Interval between vaccination and challenge	Dose of myxoma virus (ID_{50})	Local lesion		Serological Response					
					Complement-fixation tests				Neutralization tests	
					Fibroma		Myxoma		Myxoma virus	
			maximum extent	duration	before challenge	after challenge	before challenge	after challenge	before challenge	after challenge
746	1 week	10	0		0	8	0	16	0	68
747	1 week	10	0		0	4	0	16	0	61
748	1 week	10	0		0	0	0	4	9	61
749	1 week	10^3	9-erythema	transient	0	8	0	16	18	80
750	1 week	10^3	10-erythema	transient	4	16	16	64	0	95
751	1 week	10^3	5-erythema	transient	4	16	32	32	0	92
752	2 weeks	10	20, 10	three weeks	4	16	0	256	56	99
753	2 weeks	10	30, 23, 10	three weeks	8	32	32	256	9	95
754	2 weeks	10	6-nodule	transient	16	16	8	8	70	93
755	2 weeks	10^3	20, 14, 6	three weeks	8	16	16	32	74	95
756	2 weeks	10^3	22, 17, 12	three weeks	32	32	32	256	68	96
757	2 weeks	10^3	25, 15, 11	three weeks	128	64	16	512	88	97

Symbols as in Table 1.

TABLE 3.

The size of the local lesion (maximum diameters in millimetres) at the inoculation site at various intervals after the intradermal inoculation of adult rabbits with 10^3 ID_{50} of Boerlage fibroma virus.

Rabbit No.	Skin lesion					
	5 days	7 days	14 days	21 days	28 days	35 days
376	29×17 red	39×25 black centre	40×40 well defined nodule ulcerated	33×33 regressing	shallow ulcer	healed
377	15×15 red	25×25	43×43 pedunculated lump ulcerated	41×41 regressing	lump sloughing off	healed
378	18×18 red	35×20	40×40 pedunculated lump ulcerated	21×21 flat ulcer	almost healed	healed
379	18×12 red	27×19	33×21 well defined nodule ulcerated	28×18 regressing	shallow ulcer	healed
380	15×15 red	30×19	34×23 well defined nodule ulcerated	27×27 regressing	almost healed	healed
381	17×17 red	26×26	39×33 raised, ulcerated	33×25 regressing	shallow ulcer	healed

at the site of myxoma virus inoculation. In no case did secondary lesions or eye signs develop. Sera were collected 14 days after the challenge inoculation. The serological responses showed a reasonably close correlation with the clinical reactions. Some increase in titre was expected even if there had been no challenge inoculation, for antibodies appear rather slowly after infection with fibroma virus (Shaffer, 1941). Nevertheless, it is clear that considerable increases in both complement-fixing and neutralizing antibodies occurred only in those rabbits in which there was a definite response at the site of the myxoma virus inoculation. None of these rabbits could be kept for testing several months later, but the relatively high antibody titres suggest that protection would have been more prolonged than was observed after the inoculation of OA fibroma virus alone.

Experiments with the Boerlage strain of fibroma virus.

Protection with OA fibroma virus alone was not persistent enough to justify its use as a routine prophylactic measure. Inoculation with OA fibroma, followed two weeks later by myxoma virus, produced an antibody response which suggested that immunity would be prolonged, but the deliberate introduction of virulent myxoma virus into a rabbit colony is obviously an undesirable procedure. Preliminary experiments with the Boerlage strain of fibroma virus showed that it produced much larger tumours at the inoculation site and considerably higher antibody titres than those found after infection with the OA strain. A long term experiment was therefore carried out to determine the rate of appearance and the size of the lesions produced by a standard dose of about 10^3 ID_{50} of Boerlage fibroma virus, the antibody production stimulated by this, and the durability of the immunity to myxomatosis.

Six adult rabbits were inoculated intradermally over the left flank with about 10^3 ID_{50} of Boerlage fibroma virus. The size of the resultant tumour was regularly observed, and the titre of various antibodies determined. The results are shown in Tables 3 and 4.

The local reaction to inoculation of the Boerlage strain is much more severe than the reaction to OA fibroma virus, but we have never observed any signs of general disturbance of the health of the inoculated animal, even in animals inoculated in as many as forty sites for virus titrations, or in animals given massive and extensive subcutaneous inoculations such as are described in the Appendix. A large fleshy lump developed within a few days of the inoculation, reached its maximum size in about two weeks, and then regressed or separated by ulceration. The site was always healed by the end of the fifth week.

Corresponding to the greater severity of the local lesion, there was a much more vigorous antibody response after the inoculation of Boerlage fibroma virus than with the OA strain.

TABLE 4.

The development of antibodies to fibroma and myxoma viruses after the intradermal inoculation of adult rabbits with 10^3 ID_{50} of Boerlage fibroma virus.

Period after vaccination	R376			R377			R378			R379			R380			R381		
	* F-CF	* M-CF	* M-N	F-CF	M-CF	M-N	F-CF	M-CF	M-N	F-CF	M-CF	M-N	F-CF	M-CF	M-N	F-CF	M-CF	M-N
0 days	—	—	..	—	—	..	—	—	..	—	—	..	—	—	..	—	—	..
10 days	—	—	27	—	—	27	—	—	53	—	—	35	—	—	21	—	—	47
14 days	10	10	92·2	—	—	53	15	10	96·9	20	10	93·8	8	8	47	—	—	53
3 weeks	60	40	93·8	40	20	95·5	20	15	97·1	35	10	98·3	15	8	90	20	8	95
4 weeks	240	80	95·3	80	60	96	30	20	97·3	60	30	97	15	8	91·3	40	10	93·2
5 weeks	240	80	93·6	80	60	94·2	40	30	96·2	160	30	97·5	15	8	96·7	40	15	95·6
6 weeks	120	80	93·8	120	70	98	35	30	95·8	80	20	95	20	10	97	25	15	94·6
7 weeks	60	40	96·6	80	60	96·7	40	30	98·6	30	20	96	15	8	95·5	25	15	96·9
8 weeks	60	40	95·8	80	60	94·4	40	30	97·5	30	20	94·4	15	10	95·8	25	15	95·9
12 weeks	40	25	95·7	50	30	97·5	30	20	96·7	15	10	94·8	10	8	96·9	20	10	97·3
16 weeks	30	15	91·5	50	30	98·3	30	—	96·8	20	10	94·3	10	—	94·2	25	15	93·4
20 weeks	30	15	91·9	40	25	98·7	10	—	95·1	10	10	92·7	10	—	92·1	15	15	93·3
24 weeks	25	10	94	30	25	97·2	10	—	98·5	10	10	95·2	10	—	96	15	15	93·1
28 weeks	20	10	93·6	30	25	98·1	10	—	94·4	10	7·5	91·5	10	—	92·9	15	10	93·1
32 weeks	20	10	90	25	25	91·2	15	—	92·1	10	7·5	90·7	10	—	94·5	15	10	92·4
36 weeks	20	10	89	20	20	92·5	15	—	91·2	10	7·5	90·7	10	—	95·9	15	10	94·3
40 weeks	15	10	80·6	15	—	87·4	15	—	79·1	10	5	86·8	5	—	73·4			
44 weeks	15	10	84·4	12·5	—	79·7	12·5	—	88·2				5	—	81			
48 weeks				10	—	80	5	—	84				..	..	75·9			
52 weeks				5	—	84							<5	—	62			

* F-CF, M-CF=complement-fixation with fibroma (F) or myxoma (M) antigens. Figures represent reciprocal of serum dilution at which 50 p.c. haemolysis occurs.

* M-N=myxoma neutralization. Figures represent percentage reduction in pock count on chorioallantoic membrane.
—=negative. . .=test not done.

The titre of complement-fixing antibody was invariably higher with the fibroma than with the myxoma antigen, especially at the peak levels, which occurred four to six weeks after the fibroma inoculations. In both these features, the higher titre with the homologous antigen and the rather slow rise to peak titre, our results confirmed those of Shaffer (1941). In the series of animals represented in Tables 3 and 4 complement-fixing antibody to the fibroma antigen was detectable at a low titre for many weeks after the inoculation.

Neutralizing antibodies to fibroma virus were not determined, owing to lack of experimental rabbits, but the myxoma neutralizing antibodies reached their maximum titre by the third or fourth week and declined very slowly thereafter, a significant reduction in the pock count being recorded as long as a year after vaccination. Four of the six rabbits died from non-specific causes between the 36th and 52nd week after vaccination, and serial observations could be continued for 52 weeks on two rabbits only.

The more vigorous and persistent antibody response obtained with the Boerlage strain gave promise of more durable immunity to challenge infection with myxoma virus than had been obtained with the OA strain, and experiments conducted over a period of twelve months have borne this out. Twenty adult rabbits were inoculated intradermally on one flank with the same dose of Boerlage fibroma virus as that used in the foregoing experiments (10^3 ID_{50}), and at regular intervals some were challenged by the intradermal inoculation of small doses of myxoma virus. The results are shown in Table 5.

The long persistence of a high degree of immunity to challenge with small doses of myxoma virus is readily apparent. In only one out of the nineteen animals included in this experiment (R389, tested 16 weeks after vaccination) did signs of generalized myxomatosis occur. In all other animals, even those tested twelve months after the fibroma inoculation, there was only a local response at the inoculation site. In ten other animals vaccinated with the Boerlage strain of virus at different ages and tested about six months after vaccination a similar high degree of immunity was demonstrated.

It is apparent from Table 5 that the titres of complement-fixing antibodies before challenge inoculation followed much the same pattern in these animals as in those systematically examined at monthly intervals (Table 4). There were, however, discrepancies in the neutralizing antibody tests. These remained relatively high throughout the experimental period in the six rabbits listed in Table 4, but were often quite low after the 20th week in the group listed in Table 5. Simultaneous retitration of all these sera indicated that the difference was a real one. Nevertheless, rabbits with quite low titres of myxoma neutralizing antibody exhibited a high degree of immunity on challenge inoculation. Production of a local lesion at the site of inoculation of myxoma virus was followed by rises in the titre of all the antibodies measured. The titre of complement-

TABLE 5.

The clinical and serological responses in rabbits challenged with a single small dose of myxoma virus at intervals after vacoination with a single inoculation of 10^3 ID_{50} of the Boerlage strain of fibroma virus.

Rabbit No.	Clinical response: Interval between vaccination and challenge (weeks)	Clinical response: challenge dose	Clinical response: Local lesion	Clinical response: Secondary lesion or eye lesion	Serological tests: Complement-fixation, Fibroma, before challenge	Complement-fixation, Fibroma, after challenge	Complement-fixation, Myxoma, before challenge	Complement-fixation, Myxoma, after challenge	Neutralization, Myxoma virus, before challenge	Neutralization, Myxoma virus, after challenge
									p.c.	p.c.
386	8	5 ID_{50}	nil		60	60	30	30	90	90
		50 ID_{50}	14	nil		100		120		91·5
387	8	5 ID_{50}	10	nil	60	60	40	40	89	91
388	16	10 ID_{50}	14	nil	50	40	30	30	91·6	91·4
389	16	10 ID_{50}	46, 32 severe	severe conjunctivitis	10	640	10	3820	90·4	97·3
385	20	10 ID_{50}	7	nil	15	240	0	240	59	91·4
390	20	10 ID_{50}	nil		15	15	0	0	67	78
		100 ID_{50}	17	nil		240		480		97·2
391	24	10 ID_{50}	28, 33	nil	10	200	0	480	0	98
392	24	10 ID_{50}	11, 28	few	10	320	0	480	10	98·8
395	28	10 ID_{50}	nil		40	40	0	0	0	0
		100 ID_{50}	13, 8	nil		160		320		97·7
396	28	10 ID_{50}	3	nil	10	40	0	50	0	98·3
398	32	10 ID_{50}	30, 35	few	10	240	0	960	0	94·2
399	36	10 ID_{50}	28, 27	nil	10	640	0	640	14	95·7
400	40	10 ID_{50}	nil		40	50	30	30	25	32
		100 ID_{50}	11, 8	nil		..		..		96·5
403	44	10 ID_{50}	7, 6	nil	10	2560	0	1920	25	97·2
401	48	10 ID_{50}	nil		12·5	10	0	0	10	60
		100 ID_{50}	16, 16	nil		640		960		96·9
402	48	10 ID_{50}	30, 30	nil	5	240	0	640	60	99·1
404	52	10 ID_{50}	nil		15	15	15	10	62	44
		100 ID_{50}	12, 10	nil		60		160		95
377	52	10 ID_{50}	nil		5	5	0	0	84	0
		100 ID_{50}	5, 8	nil		120		160		95·2
380	52	10 ID_{50}	16, 16	nil	<5	120	0	480	62	94·6

Symbols as in Table 1.

fixing antibody was now invariably higher with the myxoma than with the fibroma antigen, a reversal of the situation existing before challenge inoculation (Table 4).

In seven of the nineteen animals the first inoculation of an estimated dose of about 10 ID_{50} of myxoma virus failed to produce any recognizable lesion, or any change in the antibody titres. In all such cases the inoculation of 100 ID_{50} a week later produced a localized nodule, and a well-defined rise in the antibody levels. The level of immunity had apparently been high enough to prevent completely the response to the first small dose of myxoma virus. This point was tested systematically by titrating a standard preparation of myxoma virus, diluted out in five twofold steps over the range of the expected endpoint, in normal rabbits and in rabbits which had been vaccinated with either the OA or Boerlage strain of fibroma virus a few weeks or a few months earlier. The titres were determined by the Reed-Muench method, using the development of a lump at the inoculation site as the criterion of infection. The results obtained in various animals are shown in Table 6.

TABLE 6.

Titrations of a preparation of the standard laboratory strain of myxoma virus, tested in normal rabbits and in rabbits which had been inoculated with fibroma virus at various intervals previously.

Rabbit No.	Previous treatment	Response to indicated dilutions of myxoma virus					Reed-Muench titre (log_{10})
		$10^{-4.2}$	$10^{-4.9}$	$10^{-5.6}$	$10^{-6.3}$	10^{-7}	
594	normal	6*	9	10	10	10	6·4
595		8	10	10	10	10	
393	Boerlage fibroma 28 weeks earlier	2	4	3	9	9	5·6
394		3	3	7	10	9	5·8
550	Boerlage fibroma 5 weeks earlier	4	3	4	6	8	5·8
553		7	9	9	10	10	6·3
557	OA fibroma 5 weeks earlier	0	0	0	0	0	—
561		6	7	10	9	10	6·2

* 6, etc.=6 of the 10 inoculated sites showed a positive reaction.

Vaccination with fibroma virus can obviously inhibit completely the development of a local lesion after the inoculation of small doses of myxoma virus. In vaccinated animals the titration gives a flat endpoint, in contrast to the perfect Poissonian distribution of takes with myxoma virus in the normal animal (Fenner and Woodroofe, 1953). Under natural conditions a proportion of vaccinated animals would completely resist infection due to the introduction of a single small dose of myxoma virus. Similar complete protection was observed in a proportion of passively immunized young rabbits (Fenner and Marshall, 1954).

The reaction of young rabbits to vaccination with fibroma virus.

Hyde and Gardner (1939) found that fibroma virus took well when inoculated intradermally in the newborn rabbit but affected the health of the animal adversely, leading to the death of some animals. Duran-Reynals (1945) reported that when large amounts of fibroma virus were inoculated into newborn rabbits an acute lethal disease resulted. Smaller doses induced progressively growing tumours, accompanied sometimes by generalized fibromas.

Widespread vaccination of breeding stock with fibroma virus limited extensive experimentation with young rabbits, but a small number of experiments were conducted with the kittens born by unvaccinated does to determine the efficiency and safety of vaccination of young animals with the Boerlage strain of virus. The results are recorded in Table 7. When small doses of virus (10 ID_{50}) were inoculated intradermally into very young animals local tumours only resulted, and there appeared to be no interference with the general health of the rabbits. With doses comparable to those used in adult rabbits (10^3 ID_{50}) the lump produced in fourteen-day old rabbits was much smaller than that usually found in adult rabbits, and in the one rabbit challenged five months later the level of immunity was not very high. Rabbits 28 days of age and older behaved in much the same way as adult animals.

TABLE 7.

The response of young rabbits to vaccination with Boerlage fibroma virus.

Age (in days)	Dose of fibroma virus	Skin lesions 14 days	Skin lesions 21 days	Skin lesions 28 days	Challenge infection interval	Challenge infection outcome
3	10 ID_{50}	12*	15	15 necrotic	—	
3	10 ID_{50}	15	28	14 regressing	—	
7	10 ID_{50}	10	16 ulcerated	10 regressing	—	
7	10 ID_{50}	12	19 ulcerated	13	—	
14	10^3 ID_{50}	17	18 ulcerated		5 months	Severe generalized infection: recovery
14	10^3 ID_{50}	14	20 ulcerated		—	
28	10^3 ID_{50}	24	28 ulcerated	regressing	5 months	Local lesion only
28	10^3 ID_{50}	12	7	regressing	—	
42	10^3 ID_{50}	30	30 ulcerated	shallow ulcer	6 months	Local lesion only
42	10^3 ID_{50}	30	32 ulcerated	shallow ulcer	6 months	Local lesion only

*=diameter in millimetres.

DISCUSSION.

The foregoing experiments confirm those of numerous other investigators who have demonstrated cross-immunity between fibroma and myxoma viruses. The immunity was complete, in that multiplication of a small infecting dose of myxoma virus was completely prevented, for only a very brief period after the inoculation of a single dose of OA fibroma virus, but a high degree of resistance

to the general manifestations of myxomatosis was present for at least a month. Thereafter the resistance fell rather rapidly, and although the disease differed from that seen in fully susceptible rabbits, it was usually severe and sometimes fatal. Certainly the degree of protection obtained even three months after vaccination was not enough to permit rabbit breeders to rely upon vaccination with a single inoculation of OA fibroma virus as a routine prophylactic measure against myxomatosis. Prolonged protection against myxomatosis would probably result from the inoculation of a moderate dose of myxoma virus one to two weeks after the inoculation of OA fibroma virus. This method has the disadvantage that infective myxoma lesions are produced and may provide a source for the infection of non-vaccinated rabbits.

The results with the Boerlage strain were much more satisfactory from the point of view of the degree and permanence of the immunity induced. The high level of resistance to myxomatosis found in rabbits vaccinated many months earlier was particularly striking. Four of the seven rabbits challenged between 40 and 52 weeks after vaccination were not infected when inoculated with 10 ID_{50} of myxoma virus, and doses of 100 ID_{50} produced small nodules at the inoculation sites, without evidence of generalized disease. The potency and virulence of the preparations of myxoma virus were always checked, and the slight reactions must be ascribed to the high degree of resistance of the vaccinated rabbits. The low antibody titres before challenge, and the relative titres of myxoma and fibroma complement-fixing antibodies, excluded the possibility of unrecognized infection with myxoma virus in the interval between vaccination and challenge. It was not possible to test whether age alone might enhance resistance somewhat, as no normal animals as old as the vaccinated rabbits were available.

For practical purposes the intradermal inoculation of all rabbits over fourteen days of age with about 10^3 ID_{50} of Boerlage fibroma virus would appear to be a safe and effective method of protection of laboratory rabbits against myxomatosis. Freeze dried fibroma virus can be prepared from infected rabbit tissue suspended in distilled water, and in our hands such preparations have maintained their potency for at least twelve months.

SUMMARY.

The immunity to myxomatosis conferred by prior inoculation with a single dose of OA fibroma virus fell off rather rapidly. One month after vaccination a large lesion developed at the inoculation site of the myxoma virus and often a few small nodules appeared elsewhere. At longer intervals after vaccination generalization was invariable and extensive, and animals not infrequently died after suffering from modified myxomatosis.

Higher antibody levels but a localized lesion only were obtained by following the inoculation of OA fibroma virus with a small or moderate dose of myxoma virus one to two weeks later.

The Boerlage strain of fibroma virus induced larger skin tumours than the OA strain, and much higher antibody levels. A high degree of protection against generalized myxomatosis was conferred for at least twelve months after vaccination with the Boerlage strain.

For practical purposes the intradermal inoculation of rabbits over fourteen days old with about 10^3 ID_{50} of Boerlage fibroma virus constitutes a safe and effective method of protection against myxomatosis.

A method of preparing large batches of fibroma virus for vaccination is given in the Appendix.

Acknowledgments.—We are indebted to Sir Macfarlane Burnet, Director of the Walter and Eliza Hall Institute, Dr. T. E. Lowe, Director of the Baker Institute, and Professor S. D. Rubbo, of the University of Melbourne, and to Messrs. R. Renton and R. Phillip, officers in charge of the animal breeding quarters of the Walter and Eliza Hall Institute and the Baker Institute, for their collaboration in the provision of animals and in allowing access to their breeding establishments.

Appendix.

The procedure outlined below was first adopted for the preparation of large amounts of myxoma virus for distribution throughout Australia during the rabbit destruction campaigns of 1951-53. Later it was modified for the preparation of large amounts of Boerlage fibroma virus for vaccination of laboratory rabbits.

Seed virus. A single large batch of seed virus was prepared, ampouled in one-millilitre lots, and stored at −70° C. Each production batch of virus was initiated from one ampoule. In this way serial passage, which might allow the selection of variant strains, was avoided.

Production batch. The back of an adult rabbit was clipped over a large area, and the ampoule of seed virus diluted in about 60 ml. of sterile diluent. The whole of this volume of fluid was inoculated subcutaneously in such a way as to ensure its wide distribution in the subcutaneous tissue of the clipped area. Several days later (5–6 days in the case of myxoma virus, 8–10 days in the case of Boerlage fibroma virus) the animal was killed and the clipped skin reflected. The greatly thickened gelatinous subcutaneous tissue was dissected off the skin, cut into small pieces, and homogenized with 500 ml. distilled water in a chilled Waring blendor. After centrifugation the virus suspension usually had a titre of 10^8 to 10^7 ID_{50} per ml. in the case of myxoma virus, and 10^8 to 10^9 ID_{50} per ml. in the case of Boerlage fibroma virus. Both viruses could be freeze-dried with about 90 p.c. loss in activity. Virus was diluted for vaccination so that each dose of 0·1 ml. contained 10^3–10^5 ID_{50} of fibroma virus. The reconstituted myxoma virus was usually used so that each dose contained 10^3 or 10^5 ID_{50}.

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ON THE INTERACTION OF HAEMOGLOBIN WITH SODIUM AND POTASSIUM

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(Accepted for publication 9th April, 1954.)

Much work has been done on the mechanisms of electrolyte accumulation in cells, and while increasing numbers of papers are appearing on aspects of ion accumulation linked with metabolic activity, some reports have appeared on the binding of ions by purified tissue components *in vitro*. Mullins (1942) reported that myosin bound potassium to a greater extent than sodium. Stratmann and Wright (1948) found a slightly greater binding of potassium than of sodium by horse haemoglobin dialysed against salt solutions.

In the present study the interaction between alkali metal ions and the haemoglobins of horse, man and dog was investigated. Certain improvements in technique had become available in this laboratory since this preliminary report of Stratmann and Wright. A flame photometer was available for metal estimations and a more permeable cellophane allowing more rapid electrolyte equilibrium was used.

The ion binding figures obtained in dialysis of haemoglobin solutions against salt solutions were compared with titration data of haemoglobin and alkali metal hydroxides.

METHODS.

The dialysis technique was similar to that of Stratmann and Wright (1948). Small volumes (3–10 ml.) of haemoglobin (2–22 p.c.) were dialysed against a large volume (7–25 litres) of solution of sodium or potassium chlorides (5–67 mMolar) or equimolar mixtures of these salts.

The pH was adjusted by the addition of alkali hydroxide and kept to within 0·15 pH by bubbling oxygen through the solution. This also served to stir the salt solution.

At intervals of 1–2 hours samples of haemoglobin solution were analysed.

Alkali metals were estimated, using a Beckman Flame photometer, following the procedures of Wynn *et al.* (1950).

Chlorides were determined by titration with silver nitrate electrometrically. Haemoglobin concentrations were determined either as acid haematin or as oxyhaemoglobin, using extinction coefficient data. Oxyhaemoglobin content of these samples was determined manometrically by ferricyanide addition.

[1]This work was partly supported by a grant from the National Health and Medical Research Council of Australia.

Osmotic pressures of protein solutions were determined using an osmometer similar to that of Bull (1941).

Membrane potentials were determined using identical calomel half-cells.

Acid-base titrations were carried out on exhaustively dialysed protein solutions by comparing the pH after addition of measured aliquots of HCl, or alkali hydroxide, with that for pure acid or base solutions of the same concentration.

Cellophane used was either a British Cellophane Co. product or from A/B Nordisk Silke Cellulose. Both these membranes were slowly permeable to proteins of molecular weight 16,000. Water dialysed against dilute KCl solution reached equilibrium salt concentration in about 3 hours.

The haemoglobins were prepared by the method of Drabkin (1946), and the solutions were Seitz filtered and stored sterile after recrystallization.

Two types of dialysis cell were used. In one the cellophane tubing was tied to a short glass tube and stoppered. This allowed removal of aliquots from time to time for analysis—the "open" cell. In the "closed" cell the cellophane was fitted inside a rigid perforated Perspex container. The protein solution was continuous with the lumen of a narrow capillary tube vertically above the cell. This arrangement allowed the osmotic influx of water to proceed only until sufficient hydrostatic pressure was developed in the capillary to balance the protein osmotic pressure. The whole of the solution in the closed cells was removed for analysis.

RESULTS.

Figs. 1 and 2 show the course of a typical dialysis experiment. The essential features common to all the dialyses may be seen in these Figures. The data for all experiments at the conclusion of the dialysis are presented in Table 1.

pH changes. The pH fell from an initial value of 7·2 to about 7·05 in the cellophane sacs open to the atmosphere. In the closed cells the pH fall was a little less. This is to be expected because of the smaller amount of atmospheric carbon dioxide able to enter the solution during the course of the experiment.

Membrane potentials. These were not measured in all cells as they were not an essential feature of the ion-binding experiment. The duplicate values, when taken, were usually within 10 p.c. In most experiments the membrane potentials were of the order of several millivolts, and such agreement is to be expected The potentials developed across a cellophane membrane, both sides in contact with the same dilute salt solution after one side had been in contact with protein solution at acid and at alkaline pH, and washed several times, were measured. This was to determine the extent to which absorption of protein affected the membrane potential. In no case was this found to be greater than 0·3 mV.

Osmotic pressure. The measurement of osmotic pressure was not always carried out. As with the membrane potential measurements, there was found a considerable variation in absolute value. Since small changes in temperature and salt concentration of solutions will be reflected here, together with the considerable effect of surface tension differences among the solutions in the capillary tubes, the maximum variation between duplicates of some 7 p.c. is considered reasonable.

Chloride concentration. The chloride concentration in both the single salt solutions and the mixed salt solution reached a stable value after 3–4 hours.

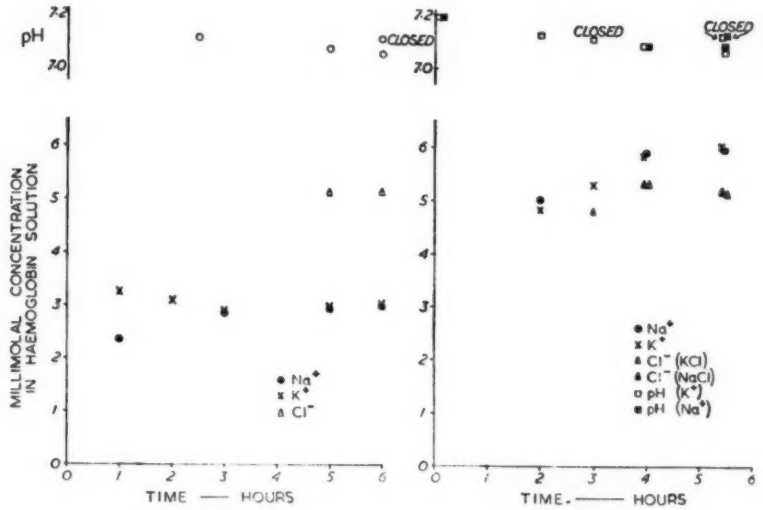

Fig. 1. Fig. 2.

Fig. 1. Showing the time course of a dialysis of 3·4 p.c. dog haemoglobin against a salt solution containing 2·7 mE/litre NaCl+2·7 mE/litre KCl at pH 7·2.

Fig. 2. Showing the time course of a dialysis of 3·4 p.c. dog haemoglobin against (a) 5·4 mE/litre NaCl; (b) 5·4 mE/litre KCl.

Alkali metal concentration. The total alkali metal concentration was the same in all three dialyses shown in Figs. 1 and 2. In Fig. 1 there is no observable difference between the potassium and sodium, either in rate of attaining equilibrium concentration, or in these concentrations. In the single salt solutions the same is seen to be true.

All ion concentrations are given as mMols./litre of water in the protein solution. The partial specific volume (0·74) of the haemoglobins was estimated pycnometrically, but as no value can be given for the water binding by haemoglobin in solution, no reliable estimate of the true solvent water space can be given.

The titration data for dog haemoglobin at 14° C. are presented in Fig. 3. The calculation of base or acid bound per molecule of haemoglobin is based on a molecular weight of 67,000 for each variety of oxyhaemoglobin (Cohn and Edsall, 1943). In 12·5 mMolar salt solution there is a slight clockwise rotation of the curve about the isoionic point. This phenomenon is more marked in other

TABLE 1.

Summary of equilibrium data from dialysis experiments.

Expt. No.	Temp. °C.	pH	Electrolyte solution: Species	Conc. mE/l.	Haemoglobin: Species	Conc. mM/l.	Hb.	Concentrations in Hb. solution mEq./litre: Calc. metal	Found metal	Calc. Cl	Found Cl	Osmotic pressure cms. H_2O	Memb. potl. mV	No. of ion detns.	Prob. of t
1	18	6·5	NaCl	30	Human	1·62	+3·1	29·7	27·9	61·6	66·2	40·8	−1·2	5	0·81
			KCl	30				29·7	27·8						
			NaCl	60	Human			58·5	55·6	61·6	66·0	41·6	−1·6	5	0·92
			KCl	60	Human			58·5	56·2	61·6	66·3	—	−1·3	5	
2	21	7·7	NaCl	33·3	Human	1·80	−23·8	39·8	38·5	55·9	64·2	—	+1·2	3	0·71
			KCl	33·3				39·8	38·3						
			NaCl	66·6	Human			79·6	78·4	55·9	63·2	—	+1·8	2	0·62
			KCl	66·6	Human			79·6	77·6	55·9	63·0	—	+1·7	2	
3	21	7·7	NaCl	33·3	Human	2·86	−38·3	44·3	43·25	50·3	51·8	—	+5·0	7	0·51
			KCl	33·3				44·3	42·9						
			NaCl	66·6	Human			88·6	87·0	50·3	50·0	—		4	0·83
			KCl	66·6	Human			88·6	89·5	50·3	52·0	—	+5·5	4	
4	37	7·4	NaCl	12·5	Human	1·28	−16·4	17·2	16·9	18·1		—	+3·7	5	0·90
			KCl	12·5				17·2	16·9						
			NaCl	25·0	Human			34·5	33·4	18·1	20·8	—	+4·9	5	0·38
			KCl	25·0	Human			34·5	33·3	18·1	21·1	—	+3·6	6	
5	16	7·1	NaCl	12·5	Horse	0·48	−2·4	13·1	12·7	23·8	25·3	13·2	+0·5	5	0·52
			KCl	12·5				13·1	12·7						
			NaCl	25·0	Horse			26·2	25·2	23·8	25·3	15·8	+0·6	5	0·28
			KCl	25·0	Horse			26·2	25·3	23·8	25·1	16·6	—	6	
6	37	7·8	NaCl	2·5	Horse	0·57	−9·5	5·8	4·4	2·1	3·2	—	+14·2	5	0·50
			KCl	2·5				5·8	4·5						
			NaCl	5·0	Horse			11·6	9·2	2·1	3·3	—	—	5	0·28
			KCl	5·0	Horse			11·6	9·4	2·1	3·1	—	+15·8	6	
7	37	6·2	NaCl	12·5	Horse	0·54	+1·62	11·7	10·8	13·3	13·7	14·1	—	6	0·02
			KCl	12·5	Horse			11·7	11·0	13·3	13·9	—	—	6	
8	18	7·1	NaCl	5·4	Dog	0·53	−2·52	6·8	5·95	4·3	5·2	—	—	5	0·81
			KCl	5·4	Dog			6·8	6·0	4·3	5·1	18·8	—	4	
9	37	7·9	NaCl	6·25	Dog	0·65	−11·4	9·75	9·1	8·05	10·0	—	+8·6	5	0·08
			KCl	6·25				9·75	8·9						
			NaCl	12·5	Dog			19·5	18·2	8·05	10·1	—	—	5	0·01
			KCl	12·5	Dog			19·5	17·9	8·05	9·95	—	+9·4	6	
10	19	6·3	NaCl	2·55	Dog	0·51	+2·01	2·09	2·03	6·2	6·40	16·8	−5·7	5	0·61
			KCl	2·55				2·09	2·00						
			NaCl	5·1	Dog			4·18	4·10	6·2	6·37	17·3	−5·7	5	0·28
			KCl	5·1	Dog			4·18	4·06	6·2	6·39	17·5	−5·6	6	
11	14	7·2	NaCl	2·7	Dog	0·51	−2·52	3·38	3·00	4·33	5·14	16·2	—	4	0·70
			KCl	2·7				3·38	3·03						
			NaCl	5·4	Dog			6·74	5·95	4·33	5·20	17·0	+2·3	5	0·67
			KCl	5·4	Dog			6·74	5·99	4·33	5·11	—	+2·2	5	

titrations carried out at higher ionic strength, and has been analysed in considerable detail by previous workers (Cohn and Edsall, 1943).

From this curve, and others carried out at different temperatures for the three haemoglobins studied, an estimate can be made of the number of metal ions present in a protein solution of given concentration at a given pH. It is seen that there is no significant difference among the curves using lithium, sodium or potassium hydroxides for the titration. The titration data confirm the fact derived from the dialysis experiments that there is no preferential accumulation by haemoglobin, that is, binding at the protein surface, of any one of the alkali metals studied.

Table 1 summarizes the equilibrium data from dialysis experiments and correlates the dialysis data with those derived from the titration curves.

At a given pH the amount of base "bound" by the protein can be taken as the base concentration in the protein solution minus the base concentration in water (or neutral salt solution) at the same pH. The equivalence of haemoglobin ions in the solutions used in the dialysis experiments have been calculated and are listed in Table 1. The expected metal concentrations in each haemoglobin solution based on these equivalents are also listed together with the chloride concentrations expected from the Donnan formula.

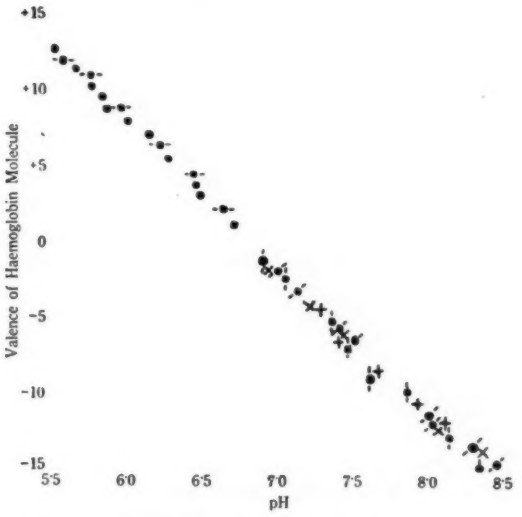

Fig. 3. Showing titration data for dog haemoglobin at 14° C. Haemoglobin concentration 3·45 p.c.

- ● HCl—no added salt
- -●- HCl in 12·5 mM. NaCl
- ↓ NaOH in 12·5 mM. NaCl
- × KOH—no added salt
- \+ LiOH—no added salt
- ↙ NaOH—no added salt

$$\frac{M_i}{M_o} = \frac{Cl_o}{Cl_i}$$

using the calculated metal concentrations, M_i and M_o in the protein solution and in the electrolyte solution respectively.

Some discrepancy is seen between the calculated and observed values of ions of both charges. While the cation concentrations observed in the protein solution after dialysis are lower than the values expected from the titration data, there is no significance between the sodium and the potassium values in the mixed or single electrolyte dialysates. The chloride values in the dialysis experiments are uniformly higher than the values calculated from the titration data.

Table 1 shows the probability of Student "t" for each set of dialysis data. These figures indicate that the probability is very small that the sodium and potassium concentrations in the haemoglobin solutions at the end of dialyses under the same experimental conditions are significantly different. The mean SD_{Na} and SD_K of all equilibrium values are 1·55 p.c. and 1·59 p.c. of the respective means. The overall inaccuracy in experimental technique would mask any true difference less than about 1·2 p.c. at the P=0·05 level.

DISCUSSION.

The essential result of these experiments is the apparent equivalence of sodium and potassium in their interaction with haemoglobin. The differences between these (hydrated) ions in solution lie mainly in diameter, ionic mobility and ionic potential (at the periphery of the charged ion). In certain instances sodium and potassium exhibit considerably different properties in their ability to bind other (generally complex) ions. In such compounds as the cobaltinitrites the size of the unhydrated potassium ion is an important factor in the formation of the crystal lattice. Pauling (1939) has pointed out that sodium, being the smaller unhydrated ion, should have a tendency to form undissociated complexes. Complexes involving un-ionized alkali metals are rare, but are certainly more common with decreasing atomic number.

In the experiments presented here there is no evidence from osmotic data, within the rather wide experimental error of these measurements, of complex formation with either metal. The findings of Stratmann and Wright (1948) must therefore be due to some other factor. Most probably they are due to bacterial contamination against which rigid precautions were not taken. Horse and human haemoglobin were infected with *B. subtilis* and Sarcina and dialysed against equimolar KCl and NaCl (30 mM each) for 24 hours at room temperature: these values in the horse haemoglobin were 30·1 mM K and 27·5 mM Na, and in the human haemoglobin 30·1 mM K and 28·5 mM Na.

There is the possibility that ion exchange resin properties exist in the haemoglobin molecules. These molecules are very much smaller than the polymerized molecules usually found in resins, and from X-ray diffraction data (Boyes-Watson *et al.*, 1947) it is probable that the anionic groups are more exposed than in most resins. There is no significant difference in the concentrations of these metals in the protein solutions under identical conditions and any ion exchange resin effect must be extremely small.

If we consider that the titration experiments provide valid data concerning the base-binding capacity of haemoglobin at a given pH, the dialysis experiments show a consistently smaller quantity of cation to be held by the protein. In general, the titration data were obtained with slightly more dilute solutions than those used for the dialyses. There is lack of quantitative agreement on reports of the proportion of solvent water in protein solutions, and it is likely that some of this discrepancy could be explained if the activities of the metal ions and of water in the solutions were known.

The marked increase in chloride concentration in the dialysis experiments compared with that predicted from titration data is a more complex problem. The protein solutions used were taken to be salt-free for titration purposes when the alkali metal had been removed to a concentration of 0·3 mEq./litre by dialysis. From the work of Adair (1949), it would appear that considerable amounts of chloride are bound by haemoglobin even on the alkaline side of the isoionic point. This chloride occupies sites near positively-charged groups (the number depending on the pH) and does not appear to affect the binding of cations.

Table 1 shows that within the limits of the experimental error there is no difference between sodium and potassium in their behaviour towards haemoglobin, nor are there any differences among the three haemoglobins used. Horse and human red cells contain haemoglobin in association with high potassium concentrations while dog red cells have a low potassium content. The evidence for preferential potassium binding by cell constituents is extremely scanty and it appears that some other mechanism controls the ion distribution. Steinbach (1950) has produced evidence that the sodium and potassium partition between centrifuged cell debris and the supernatant solution is in the direction of a slight but definite accumulation of sodium in the debris. This finding lends weight to a proposal by Dean (1941) that a "sodium pump" mechanism in the cell membrane is able to bind sodium and move this ion across the cell boundary.

The photometric method of metal estimation is undoubtedly superior to the chemical methods used in the previous study. The latter are subject to ammonium contamination which may easily occur during the long period required for dialysis with less permeable cellophane than used in the present series, and are temperature sensitive. Room temperatures vary considerably in this laboratory.

SUMMARY.

The interaction between alkali metals and haemoglobins of horse, man and dog has been studied.

Titration of the proteins with sodium, potassium and lithium hydroxides yielded pH values for given metal and protein concentrations identical in all cases.

On dialysis of protein solutions against sodium and potassium chloride solutions there is no observed difference between these ions in osmotic pressures produced or in metal concentrations in the protein solutions.

There is no evidence for preferential binding by the haemoglobins of either of the ions studied. There is no evidence for any differential alteration in ion activities in the protein solutions.

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DETERMINATION OF LIPID PHOSPHORUS IN ULTRA-MICRO QUANTITIES OF SERUM

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(Accepted for publication 23rd April, 1954.)

Analysis of changes of blood lipid in the rat is complicated by the small amount of blood available for frequent serial analyses, and by the low concentration of lipid in the blood of this animal (Boyd, 1943). Ultramicro procedures are available for measuring some lipid fractions in the isolated state (Schmidt Nielsen, 1942; Givor and Kirk, 1953; Grunbaum, Schaffer and Kirk, 1953). A limited amount of information is available concerning extraction and separation of various lipids from milligram samples of tissues. Schmidt Nielsen (1944) digested 1 mg. samples of tissue in potassium hydroxide prior to separation of saponifiable from non-saponifiable lipids. Billing, Conlon, Hein and Schiff (1953) determined total lipid, lipid phosphorus, total fatty acids (and iodine number) in a single needle biopsy sample of liver weighing several milligrams.

In this paper procedures for determination of lipid phosphorus in microlitre quantities of serum are reported. This work was carried out in connection with the development of a method for determination of the major lipid factors in a single 15 μl. aliquot of rat serum, and the methods have been specifically designed to ensure rapid determination of the lipid phosphorus fraction. While this work was in progress, Schaffer, Fong and Kirk (1953) reported a method for estimation of total and inorganic phosphorus in 1 mg. samples of liver. These workers used a rather more elaborate procedure for phosphorus determination than that reported in this paper.

MATERIALS.

Apparatus used to separate microgram quantities of lipid phosphorus from non-lipid phosphorus.

10 μl. pipettes. Various stages in their construction are shown in Fig. 1 and are described in the Appendix.

Rack and pinion. A glass capillary tube containing serum is held in the groove of the head of the moveable arm of a rack and pinion (Fig. 2) by a rubber band. During pipetting, the 10 μl. pipette is held in a fixed position and the serum sample in the capillary tube raised into contact with the pipette tip. The serum is drawn into the pipette by suction. A resumé of the principle of fixed pipetting is given by Glick (1948).

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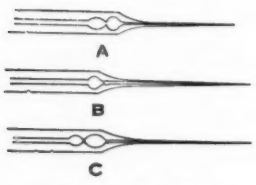

Fig. 1. See text.

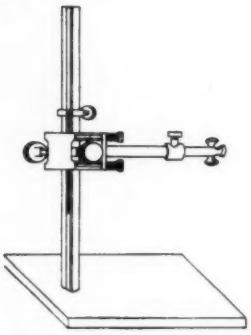

Fig. 2. Rack and pinion.

1 ml. volumetric flasks. Vessels reproduced diagrammatically in Figs. 3 and 4 are prepared from hard glass tubes, 1 mm. wall thickness, 8 mm. bore. The vessels are so made that the 1 ml. graduation mark is placed several millimetres below the centre of the narrow constriction. After making up the contents of a flask to volume, a drop of the contents is thrown into the centre of the narrow constriction. The air bubble so formed is used to mix the contents of the flask.

0·20 ml. volumetric flasks. These are prepared from hard glass tubes, 1 mm. wall thickness, 4 mm. bore, in a similar fashion to the 1 ml. volumetric flasks. The graduation mark is placed about 1 mm. below the centre of the constriction.

Centrifugation of volumetric flasks. Bakelite rods 2 cm. diameter are fashioned to fit into the cups of the angular head (Cat. No. 295) of the multispeed attachment of an "International" refrigerated centrifuge. A hole is drilled along almost the length of the axis of each rod to hold a 1 ml. flask. If rubber tubing sleeves are inserted into the holes, then the 0·20 ml. flasks may be used in the same rods.

Spectrophotometry in microgram procedure. Beckman model DU spectrophotometer, slit width 0·1 mm., 830 $m\mu$ transmission using a 1·1 mm. hole in the diaphragm attachment and 1·0 ml. cuvettes (Pyrocell Manufacturing Co.). Possible decrease in precision of absorbance measurements due to use of the microcuvettes may be minimized by the following precautions. The alignment of the cuvettes is frequently checked by noting the absorbance with each cuvette in its normal position and the plateau absorbance (Kinsey, 1950); matched cells are used, and care is taken to remove numerous small bubbles which form in the heteropoly solution in contact with the silica. A description of the adjustment of the spectrophotometer for use with the microcells and diaphragm is given by Kirk (1950). The spectrophotometer is standardized at 100 p.c. transmission against a reagent blank. Absorbance of the heteropoly blue solution should be read within 12 hours of colour development as recommended by Boltz and Mellon (1947).

Standard phosphorus solutions. 2·19 gm. of KH_2PO_4 (AR) are dissolved in 500 ml. of water. This solution contains 1·0 mg. of phosphorus/ml. Other standard solutions may be prepared from it by dilution as required. A drop of chloroform is added to each standard as preservative.

Hydrazine sulphate-sodium molybdate mixture. 0·075 gm. of hydrazine sulphate (AR) is dissolved overnight in 50 ml. of 1 N perchloric acid (AR). 0·31 gm. of crystalline sodium molybdate (BP) is dissolved in 1 N perchloric acid immediately prior to the addition of 10 ml. of the hydrazine sulphate solution and the mixture made up to 50 ml. with 1 N perchloric acid. The mixture should be used within one hour of preparation.

METHODS.

Preparation of extracts.

Method I. Acid insoluble phosphorus procedure. About 0·8 ml. 5 p.c. trichloroacetic acid is introduced into a 1 ml. volumetric flask which is then held in the arm of the rack and pinion. Serum is drawn into a 10 μl. pipette and the tip of the pipette introduced into the trichloroacetic acid to a depth of about 1·5 cm. The vibrator unit is switched on and the shaft brought into contact with the capillary tip of the pipette (Fig. 3).

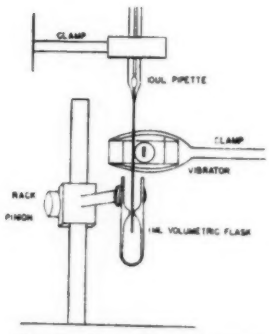

Fig. 3. Dispersion of serum proteins in trichloroacetic acid.

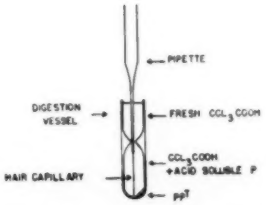

Fig. 4. Removal of acid-soluble phosphorus.

If the pipette is firmly clamped, the tip of the pipette undergoes intense vibration without visibly-associated vibration of the body of the pipette. In a well-made pipette the centrifugal action of the serum in the vibrating capillary tip will automatically discharge the serum from the top constriction from where it flows to the lower constriction. If automatic discharge of serum does not occur, then slight pressure will bring it about. The serum proteins are precipitated as an opalescent suspension and are washed relatively free of acid-soluble phosphate by dilution. The volume of trichloroacetic acid is made up to 1 ml. and the contents of the flask are mixed and spun (20,000*g*.) for 15 minutes. The flask is then held on the movable arm of the rack and pinion and the hair capillary tip (about 2 cm. long) of a pipette is introduced into the flask. The precipitate lies to the side of the lower part of the flask and the hair capillary should touch the bottom of the flask (Fig. 4). The pipette is attached to a water suction-pump and the supernatant drawn off. The vessel and the precipitate are partially washed by adding fresh trichloroacetic acid to the flasks during withdrawal of the supernatants. The supernatant should be removed at a rate of less than 1 ml. in 3 minutes in order to avoid disturbing the precipitate. The precipitate is dried *in vacuo*.

Method II. Solvent microgram phosphorus procedure. Using the technique described in the trichloroacetic acid method, 10 μl. of serum are discharged into a 200 μl. volumetric flask containing about 180 μl. of a mixture of ethanol-dioxan (2:1). The mixture is made up to 200 μl. with more solvent mixture. The flask is capped with a small length of rubber tubing closed at one end with staples previously fixed in position with an ordinary office stapling machine. If thought necessary, saturation of the air in the flask with solvent from an atomiser prior to capping will reduce any decrease in solvent mixture due to evaporation. Ninety p.c. of the solvent may be withdrawn, using a constriction pipette with hair capillary

tip (Fig. 5), and transferred to a 1 ml. volumetric flask. The solvent is evaporated off (Fig 6a). The lipid phosphorus is contained in the residue.

Fig. 5. 180 μl. pipette.

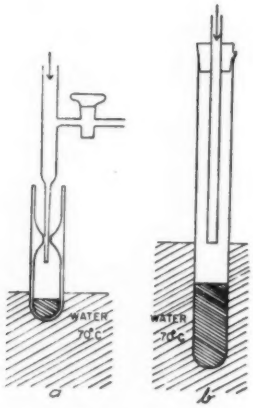

Fig. 6. Evaporation of lipid phosphorus solvent; (a) microgram procedure; (b) submilligram procedure. Air passes into vessels in direction of arrow.

Conversion of lipid phosphorus to inorganic phosphorus and colour development. To the dry serum proteins from Method I, or the residue from Method II, is added the equivalent of 62 μl. of 10·0 N perchloric acid.[2]

The tube is lowered into an oil bath to a depth of about 5 mm. The temperature is raised to 220° C. and maintained at this temperature until the perchloric acid is clear (about 15 min.). 0·34 ml. of distilled water is then rapidly added from a graduated 1 ml. pipette which terminates in a capillary (as shown in Fig. 7) so that the water thoroughly mixes with the digest. The mixture is made up to volume with freshly-prepared hydrazine sulphate-sodium molybdate mixture and contents of the flask mixed. The flask is placed in a boiling water bath for five minutes and is then rapidly cooled in a beaker of cold water. The heteropoly solution is remade to volume with distilled water and its absorbance measured. A reagent blank is similarly prepared.

Phosphate standards are prepared by pipetting three 10 μl. aliquots of a standard phosphate solution containing 50 μg. phosphorus/ml. into three 1 ml. flasks with the pipette used for delivery of serum. The absorbance corresponding to this concentration of phosphorus will be found to be practically constant at the different times when standards are prepared. When the

[2]In the experimental work reported in this paper 11·7 N (70 p.c.) perchloric acid was used. Titration of acid remaining after digestion showed a negligible loss of less than 5 p.c. However, in order to increase the volume of the small quantities of acid, 10·0 N (60 p.c.) perchloric acid is recommended.

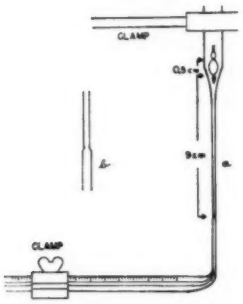

Fig. 7. Diagrammatic representation of calibration of a microlitre pipette as described in the Appendix.

operator has assured himself on this point, the phosphorus unknowns may be read off from the calibration curve without preparation of further standards.

The value depends partly on the individual Beckman Spectrophotometer in use (Ewing and Parsons, 1948; Cannon and Butterworth, 1953; Kemmerer, 1946) and is not transferable. This procedure makes it unnecessary to calibrate exactly the pipette used to measure the standard and unknown solution.[3]

Submilligram procedure. Two ml. of serum are dispersed dropwise in 45 ml. of 2:1 ethanol ethyl-ether and the mixture refluxed in an atmosphere of carbon dioxide on a 70° C. water bath for 1 hour. The solution is cooled and made up to volume with ether in a 50 ml. volumetric flask. The proteins are allowed to settle, the solution is filtered and 10 ml. of the filtrate run into a digestion vessel. The solvent is rapidly removed in a stream of air (Fig. 6b) and the equivalent of 3·1 ml. of 10 N perchloric acid added. The vessel is placed on a sand bath with a deep temperature of 300° C. and the residue-covered glass occasionally swilled with perchloric acid. Digestion is complete within 30 minutes. The digest is washed into a 50 ml. long-necked volumetric flask followed by 20 ml. of freshly prepared hydrazine sulphate-sodium molybdate mixture and the volume made up to 50 ml. with distilled water. A reagent blank is also prepared. The contents of the flasks are mixed and placed for 5 minutes in a boiling water bath[4], and then rapidly cooled in a cold water bath.

Phosphate standards are prepared by adding 1 ml. of phosphate standard containing 25 μg. of phosphorus to two 50 ml. long-necked volumetric flasks followed by the equivalent of 3·1 ml. of 10 N perchloric acid. The mixture is diluted with water. Twenty ml. of freshly prepared hydrazine sulphate-sodium molybdate mixture are added and the volume made up to 50 ml. with water. The heteropoly blue is developed in a boiling water bath and its absorbance measured.

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[3]Serum leaves a layer of protein on the glass which may be removed by leaving the pipette filled with near-saturated potassium hydroxide. If the protein layer is not removed, the standard solution may fail to wet completely the glass and air bubbles will be trapped in the solution.

[4]A 10 litre saucepan with lid is a very convenient water bath.

RESULTS.

Adherence of absorbance to Beer's law over the desired range of phosphorus concentration was assessed from measurements made over a period of several months on standard phosphate solutions. All measurements of the heteropoly blue were made on solutions at the prevailing room temperature. Data for the submilligram procedure are listed in Table 1. In Tables 1 and 2 the variance was computed from the absorbance values ×1000.

TABLE 1.

Relation of phosphorus concentration to absorbance and variance—submilligram procedure.

Phosphorus concentration	Number of samples	Absorbance ×1000 variance	mean
μg./50 ml. heteropoly blue solution	(N)	(σ_i^2)	
6·25	6	1·8	111
12·5	10	3·1	222
25	10	0·77	440
50	11	34	876

Data obtained from estimation of the phosphorus content of the phosphorus standard solutions by the microgram procedures are listed in Table 2. The vibrator unit was not used in these estimations.

The data in Tables 1 and 2 show that Beer's law (linear regression of absorbance on concentration) is obeyed over the absorbance range 0·10 to 1·0.

TABLE 2.

Relation of phosphorus concentration to absorbance and variance—microgram procedure.

Phosphorus concentration	Number of samples	Absorbance ×1000 variance	mean
μg./1 ml. heteropoly blue solution	(N)	(σ_i^2)	
0·15	10	74*	129
0·30	9	82†	257
0·60	10	16	507
1·20	10	170	1020

Correlation coefficient = 1·000.
*with an outlying value omitted $\sigma_i^2 = 11$ (N = 9)
†with an outlying value omitted $\sigma_i^2 = 14$ (N = 8)
(see text for discussion)

As indicated in Table 2, the variance values were greatly affected by 2 apparently spurious values. The probability that these values belong to the normal population to which the rest of the values in the same phosphorus con-

centration group may be assumed to belong was computed. It was $<0{\cdot}01$ in each case.[5]

Further evidence of the spurious nature of these values is the absence of extreme deviations between the various paired values discussed below.

The absorbance of a number of the heteropoly blue solutions was measured at 1 and 24 hours after colour development. There was, in general, a slight decrease by 24 hours. To estimate its significance, the mean of each pair of values was computed, and the difference between each pair was expressed as a percentage of the mean for the pair. The mean decrease $\pm\sigma_{md}$ obtained by the submilligram procedure $=1\pm0{\cdot}3$ p.e. $p<0{\cdot}01$ (25 pairs of values); and by the microgram procedure, $2{\cdot}7\pm0{\cdot}7$ p.e. (mean decrease of 20 pairs of values), $p<0{\cdot}01$. The percentage decrease appeared on inspection to be independent of phosphorus concentration.

Lipid phosphorus.

At the time the phosphorus standards were analysed, serum was analysed for lipid phosphorus content by the three methods previous described. The serum was obtained from fasting male rats, 250–350 gm. weight.

Statistical analysis of mean values.

The lipid phosphorus content of each serum sample was estimated in duplicate by at least two of the three methods. In order to determine whether the various methods gave similar estimates for lipid phosphorus content of serum, the mean phosphorus values obtained by each of two methods were compared in the following manner. The respective means of the two sets of duplicate estimations made on the same serum sample were computed; $\bar{x}_1, \bar{x}_2$. These paired means were then subtracted $\bar{x}_1-\bar{x}_2$. The difference was expressed as a percentage of the mean value of the paired means;

$$\frac{2\,(\bar{x}_1-\bar{x}_2)}{\bar{x}_1+\bar{x}_2}\times 100.$$

The mean of all the percentage differences obtained from the duplicate analyses made by each of the two methods on serum samples, and the standard deviation of the mean (σ_m), were then computed and used to show the differences in the estimate of lipid phosphorus content of serum by the two methods.

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[5]The computation proceeded thus:

$$\text{"}t\text{"} = \frac{\bar{x}}{\left(\frac{\Sigma x^2}{N\cdot 2}+\frac{\Sigma x^2}{(N\cdot 2)\ (N\cdot 1)}\right)^{\frac{1}{2}}}$$

$P=N$. probability corresponding to "t" as shown in Tables for "t" of Fisher and Yates, 1948.

The acid-insoluble phosphorus value was 2·2±1·0 p.c. (mean and σ_m of differences) below the alcohol-ether value. Range, —5·7 to 2·7 p.c. of the paired, alcohol-ether value.

The solvent microgram phosphorus value was 0·33±0·6 p.c. (mean and σ_m of 7 differences) below the alcohol-ether value. Range, —2·3 to 3·1 p.c. of the paired alcohol-ether value.

The acid-insoluble phosphorus value was 2·2±1·0 p.c. (mean and σ_m of 8 differences) below the solvent microgram phosphorus value. Range, —5·0 to 1·4 p.c. of the paired microgram solvent value.

The capacity of a method to give reproducible estimates of lipid phosphorus was assessed in the following manner. The difference between duplicate estimations obtained with the use of the method on each serum sample was expressed as percentage of mean of the duplicate values for the sample. The mean and standard deviation of the percentage differences for the various samples were computed. The data for the three methods are listed in Table 3.

TABLE 3.

Difference (△) within duplicate pairs.

Method	Mean difference (%)	σi*) (%)	Range (%)	N**)
Alcohol-ether procedure	0·96	1·1	2·0	11
Acid-insoluble phosphorus procedure	2·4	2·8	5·4	8
Solvent microgram procedure	2·3	2·8	4·8	5

*) Standard deviation of individual differences within duplicate pairs, $\frac{\Sigma\triangle^2}{N}$

**) N=number of sets of duplicates.

The following data were also obtained from analysis of serum samples by the alcohol-ether control procedure.

Serum lipid phosphorus content: 4·62±0·16 mg./100 ml. (mean and σ_m of 13 sera). Decrease in heteropoly blue concentration 24 hours after colour development: 0·88±0·23 p.c. (mean and σ_m of 6 paired samples). Variance within 12 sets of duplicate pairs 1 hour after colour development =0·72, and within 5 duplicate pairs at 24 hours =0·39.

DISCUSSION.

A number of workers have extracted serum lipids with boiling alcohol-ether (Bloor, 1914; Man and Gildea, 1932), but there is evidence that the cold solvent mixture is adequate providing the serum proteins are finely dispersed in a large bulk of solvent (Stewart and Hendry, 1935; Boyd, 1936). So far as phospholipid extraction is concerned, data in this paper support this view.

The alcohol-ether extract of serum contains a number of non-lipid substances. However, it is doubtful whether the extract contains non-lipid phosphorus (Egsgaard, 1948; Man, 1937; Sacks and Van Slyke, 1953; Ellis and Maynard, 1937). This is surprising in view of the variety of phosphorus-containing substances in serum. However, the data of the workers cited seemed to show that at most only a few per cent. of the total phosphorus in the extract are not lipid phosphorus. This confusing subject has been reviewed by Witcoff (1949).

Trichloroacetic acid has been used to separate serum lipid phosphorus from acid-soluble phosphorus prior to determination of the former (Fawaz, Lieb and Zacherl, 1937; Noberg and Teorell, 1933; Zilverschmidt and Davis, 1950). Zilverschmidt and Davis (1950) found that values for acid-insoluble phosphorus and phosphorus soluble in boiling alcohol-ether extracts of serum agreed to within several per cent. Data in this paper confirm this finding.

A considerable amount of systematic examination has been made of the reaction underlying the phosphorus determination by its conversion to the heteropoly acid—phosphomolybdic acid—followed by its reduction to a blue heteropoly complex (Berenblum and Chain, 1938; Boltz and Mellon, 1947; Lowry and Lopez, 1946; Polley, 1949; Allen, 1940; Griswold, Humoller and McIntyre, 1951). This and other work, has shown that a great variety of reducing agents may be used to form the blue complex. However, wavelength of maximum light absorption, intensity of absorption at this wavelength, stability of the heteropoly blue and pH optima for the reduction mixture may vary according to the reduction procedure. To some extent the choice of reductant depends on competing requirements for simplicity of operation, sensitivity, and freedom from interfering substances. Interfering substances are not usually encountered in blood lipid-phosphorus determination and it was considered that stability of colour and simplicity of operation were requisites for accurate microgram determination of serum lipid phosphorus. It appeared that the procedure of Boltz and Mellon (1947) for determination of orthophosphate satisfied these criteria. These workers used hydrazine sulphate as reducing agent. Recourse to published data showed that this method was as sensitive as that using stannous chloride which reduces the greatest possible number of molybdic molecules in the heteropoly complex per molecule of phosphorus (Berenblum and Chain, 1938).

The procedure of Boltz and Mellon was adopted by Beveridge and Johnson (1949) for measurement of lipid phosphorus after conversion to orthophosphate in sulphuric acid. However, perchloric acid was used in the microgram procedure for the following reasons. As noted by Schaffer *et al.* (1953), the large surface to volume ratio of solutions in the capillary reaction vessels used in this type of analysis makes it particularly desirable to see that the acid wets all the sample during digestion. Perchloric acid is useful in this respect as it distils above 210° C. and regulation of the temperature of the acid between 210° C.

and 230° C. allows controlled condensation of acid, together with a flow of hot acid, over the walls of the digestion vessel above the bulk of the digest. Also, complete digestion of the organic matter as well as measurement of the small volumes of acid used, is facilitated by the fact that three times the quantity of 60 p.c. perchloric acid is used to give the same acidity in the reduction mixture as concentrated sulphuric acid, though dilute sulphuric acid has been used for digestion on an ultramicro scale (Schaffer *et al.*, 1953; Billing *et al.*, 1953). As pointed out by King (1932), oxidation with perchloric acid is smooth, more rapid and more complete than with sulphuric acid, and the use of hydrogen peroxide is quite unnecessary. Moreover, the data in this paper show that the reducing action of hydrazine is not complicated by its use in a perchloric acid medium.

The small scatter of the absorbance values about a linear regression line shows that the unknowns can be read by reference to a standard linear regression of absorbance on concentration. This procedure obviates the necessity to prepare standards with every unknown. Further simplication may be achieved by basing the microgram regression on the mean of replicate measurements of a phosphorus standard giving a transmission of about 40 p.c. where the error of spectrophotometers is minimal (Twyman and Lothian, 1933). When using the simplified procedure, the possible influence of instrumental errors on reproducibility of absorbance measurements should be considered (Cannon and Butterworth, 1953; Caster, 1951; Mandel, 1949). However, in routine work variation between phosphorus unknowns from methodological and instrumental sources may be very small compared with that due to normal variation between animals and there are often occasions when the gain in information, resulting from the increased number of determinations of phosphorus (or relevant unknowns), made possible by the use of the standard regression, more than compensates for any slight loss in precision in determination of the phosphorus unknowns. When a standard regression is used, it is essential to use freshly-prepared reagents as recommended in the text. Otherwise highly reproducible values for standard phosphorus absorbance will not be obtained.

SUMMARY.

Two methods for isolation of lipid phosphorus from ultramicro quantities of serum are described.

The lipid phosphorus is isolated as alcohol-dioxan soluble phosphorus, or trichloroacetic acid insoluble phosphorus, and converted to inorganic phosphorus in perchloric acid. The phosphate is determined by the procedure of Boltz and Mellon (1947). A macro control procedure is described. Differences in the estimates of lipid phosphorus content of serum samples obtained by the three methods were not statistically significant.

Acknowledgment. The writer wishes to thank Professor Quensel for discussion of statistical problems related to this work.

Appendix.

Construction and calibration of 10 μl. pipettes. The various stages in their construction are shown diagrammatically in Fig. 1. Thick-walled 1 mm. bore hard-glass capillary tubing is drawn out and the capillary is partially collapsed in a small oxy-coal gas flame approximately 10 mm. from the drawn end of the thick-walled tube. The pressure in the capillary is raised while the glass is still red hot, with the result that the capillary is blown into a bulb on each side of the restriction. By repeating this sequence several times a constriction is formed as shown in Fig. 1a. The glass is drawn out below the constriction to form a fine capillary tube (Fig. 1b). A second constriction is placed several mm. above the first and the adjacent capillary is blown out into a bulb on either side, as described above, to form the pipette reproduced in Fig. 1c. The top constriction is made only just small enough to hold the column of serum in the pipette against gravity. The lower constriction is made narrower. A more easily constructed pipette may be made by placing two properly formed constrictions (Linderstrom-Lang and Holter, 1940) in a capillary tube separated at an appropriate distance to give a delivery volume of about 10 μl., and the tubing is drawn out immediately below the second constriction.

Calibration of pipettes. The delivery volume for serum may be easily ascertained by displacing serum (or water) directly into a calibrated pipette (Fig. 7). The calibrated pipette is filled as far as the zero mark and the tips of the two pipettes are brought together, resulting in the union of the water phases in the pipettes. Surface tension ensures an effective seal about the junction of the pipettes. In a properly constructed pipette the surface tension in the upper constriction is unable to hold the increased head of water against gravity and the serum flows from the top constriction to the lower one. The delivery volume of the uncalibrated pipette is then read off from the increase in water content in the calibrated pipette. A commercial pipette, graduated to 1 μl., may be used as precise determination of the delivery volume of serum is unnecessary in the procedures described in this paper.

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THE INFLUENCE OF RETICULOENDOTHELIAL BLOCKADE ON HORMONE INDUCED EOSINOPENIA

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(Accepted for publication 15th March, 1954.)

A most consistent physiological effect of 11-dehydro-17-hydroxycorticosterone (cortisone) and 17-hydroxycorticosterone (compound F) is to cause a marked reduction in the numbers of circulating eosinophils. The mechanism which underlies this phenomenon has not been elucidated. One hypothesis is that these cells are sequestrated into those organs which comprise the reticuloendothelial system; and this hypothesis was tested in the following experiments in which the reticuloendothelial system was "blocked" with carbon particles (india ink) and a colloidal dye (trypan blue). The results indicate that under the conditions of these experiments, hormone (cortisone) induced eosinopenia is not prevented by reticuloendothelial blockade.

Materials and Methods.

Adult male guinea-pigs were used in these experiments. They were selected from stock and weighed between 350 and 500 gm. Their eosinophil counts ranged between 150 and 2,500 eosinophils per mm^3. of blood. Blood was collected from the foot pad of the animal as has been described by Vallejo-Freire (1951). Eosinophils were counted as has been previously described (Hudson, 1954). Before the injection of either india ink or trypan blue, eosinophil counts were performed at 3-hourly intervals during a control day to observe any diurnal fluctuation of these cells. Before the injection of either india ink or trypan blue the animals were given 25 mg. of cortisone acetate suspension (Merck) by stomach tube. The circulating eosinophils were counted before and 6 hours after the exhibition of cortisone. Any change in the number of circulating eosinophils is expressed as a percentage of the value before cortisone (Tables 1A and 1B).

TABLE 1A.*

Animal	Dose of I.V. india ink (ml.)	P.c. fall in circulating eosinophils	
		Before india ink	After india ink
19/453	3·4	90	97
20/453	3·7	95	97
26/453	5·5	97	89
12/153	4·8	94	92
30/653	6·0	96	96
31/753	1·5	97	98

[1]This investigation has been carried out in partial fulfilment of the requirements of the University of Melbourne for the degree of Doctor of Philosophy.

TABLE 1B.*

Animal	Dose of subcutaneous trypan blue (ml.)	P.c. fall in circulating eosinophils	
		Before trypan blue	After trypan blue
33/753	11·5	97	94
35/753	9·0	97	91
36/753	9·0	94	91
37/853	12·0	90	97
38/853	12·0	90	95
41/853	16·0	95	96
42/853	16·0	95	98

*Each animal received 25 mg. of cortisone acetate by stomach tube before and after the series of india ink (Table 1A) and trypan blue (Table 1B) injections.

India ink experiments.

Reeves' commercial india ink was prepared as a 20 p.c. suspension in sterile isotonic saline. The injections were given intravenously, initially into ear veins by the method of Markham and Kent (1951) using a 26-gauge needle. This was found commonly to cause venous thrombosis, and when all the ear veins had become thrombosed, the leg veins were used for further injections as described by Roth (1921). Injections were given on approximately alternate days. Individual injections varied from 0·5 ml. to 1·2 ml. The total dose varied from 1·5 ml. to 6·0 ml.

Trypan blue experiments.

Trypan blue was prepared as a 1 p.c. solution in sterile isotonic saline. One ml. of this solution was injected subcutaneously on approximately alternate days. The animals became noticeably blue after 2–3 injections, and this blueness increased with further injections. The total dose varied from 7–15 ml. of the 1 p.c. solution.

At the end of the period of injections the animals were given a further dose (25 mg.) of cortisone acetate by stomach tube. The eosinophils were counted before and 6 hours after cortisone.

After this the animals were killed by an intraperitoneal injection (1 ml.) of saturated nembutal solution. The organs were removed immediately, and sections of lung, liver, spleen, lymph node, adrenal and kidney were fixed in formol saline for histological examination.

RESULTS.

Tables 1A and 1B show the individual results in both series of experiments. It is clear that at this dose level neither trypan blue nor india ink materially influence the ability of cortisone to produce eosinopenia. In every animal of both series of experiments there is a fall in the eosinophils which is greater than 90 p.c. of the initial level, this fall differing in no way from that produced by cortisone before the animals had received india ink or trypan blue.

In those animals which had received india ink, microscopic examination showed that the reticuloendothelial cells of the liver, spleen and lymph glands were filled with particulate matter. This was interpreted as being the carbon

particles from the india ink suspension. The degree of filling of these cells with carbon particles varied with the amount of india ink the animal had received. The organs of those animals which received trypan blue were blue in colour to the naked eye, but did not reveal any remarkable abnormality to microscopic examination.

DISCUSSION.

It has been accepted that one of the important functions of the reticuloendothelial system is to remove damaged and effete blood cells from the circulation. Another function that has been ascribed to this system is the production of antibodies. Consideration of this latter function led to the concept of reticuloendothelial blockade. Thus Siegmund (1922) showed that the production of haemolysins and haemagglutinins was lessened or totally suppressed in rabbits in which reticuloendothelial blockade had been induced. Jungeblut and Berlot (1926) showed that the production of diphtheria antitoxin was reduced or abolished in guinea-pigs which had received injections of india ink. They also showed a fall in the complement titre and an impairment of the respiration of the cells of the liver and spleen following injections of india ink.

These authors ascribe this phenomenon to the inhibition of the normal antibody producing function of the cells of the reticuloendothelial system, the inhibition being induced by filling the cells with colloidal particles of india ink. Bedson (1926) showed that reticuloendothelial blockade in rabbits caused a rise in the number of circulating platelets. This rise in the number of platelets he attributed to the inhibition of the normal platelet destroying function of the reticuloendothelial system. In a general review of the relationships between the reticuloendothelial system and immunity, Jaffe (1931) cautions against the acceptance of reticuloendothelial blockade, although he admits that an alteration in immune responses can occur in adult animals, particularly if india ink is used as the blocking agent.

More recently, the concept of reticuloendothelial blockade has been investigated in relationship to hormone induced eosinopenia. Wesley (1953) showed that in rats which were given trypan blue injections the numbers of circulating eosinophils were greater in that group of rats which had received trypan blue than in the control group, although no animals served as their own controls. He observed also that the eosinopenic response to adrenaline was not abolished. In this series of experiments there was no significant difference in the numbers of the circulating eosinophils at the beginning and at the end of the experiment, as is shown in Table 2.

TABLE 2.

To show the changes in the resting levels of circulating eosinophils before and after reticuloendothelial blockade (no cortisone).

Animal	Average circulating eosinophil level (cells/mm³.) Before treatment	After treatment	Treatment
19/453	1080	1425	India ink
20/453	1115	1095	
26/453	1225	1105	
12/153	415	305	
30/653	2500	1875	
31/753	375	400	
33/753	505	610	Trypan blue
35/753	1705	930	
36/753	2685	1580	
37/853	195	175	
38/853	150	1135	
41/853	430	810	
42/853	445	345	

Essallier and Wagner (1952) induced reticuloendothelial blockade in adult guinea-pigs by the injection of trypan blue. They used a 1 p.c. solution which was injected every second day until a total dose of 6 ml. had been administered. The animals were then given 10 mg. of ACTH intramuscularly. In those animals which had been treated with trypan blue and then given ACTH there was no fall in the number of circulating eosinophils. Before treatment with trypan blue this dose of ACTH had caused an average fall of 94 p.c. in the number of circulating eosinophils. From these results these observers claim that the reticuloendothelial system must be intact if circulating eosinophils are to disappear after the giving of ACTH.

The results of this current series of experiments are fundamentally at variance with those reported by Essallier and Wagner. In none of the experimental animals injected either with india ink or trypan blue did the treatment, calculated to induce reticuloendothelial blockade, impair the ability of cortisone to produce a fall in the number of circulating eosinophils. The evidence that the function of the reticuloendothelial system is blocked must be accepted with caution. If this evidence is accepted, then reticuloendothelial blockade does not influence the ability of cortisone to cause disappearance of circulating eosinophils, nor does blockade influence their overall levels. The only distinct conclusion that may be drawn is that these results refute the claims of Essallier and Wagner that trypan blue blockade inhibits the eosinopenia due to the secretion of active adrenal steroids.

SUMMARY.

Reticuloendothelial blockade was induced in guinea-pigs by the use of india ink and trypan blue.

In animals which had been so treated there was no change in (a) the ability of cortisone to induce eosinopenia, and (b) the resting levels of circulating eosinophils before and after reticuloendothelial blockade.

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THE DETERIORATION DURING STORAGE OF RED CELL ANTIGENS

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(Accepted for publication 9th February, 1954.)

In the technique of demonstrating isoagglutination, emphasis has been, naturally enough, on the antibodies in the serum rather than on the antigen in the red cells. Nevertheless, the sensitivity of the antigens to the action of the antibodies cannot be ignored, as it is known that this sensitivity decreases as the length of storage increases. Wiener (1943) draws attention to the fact that blood cells retain their sensitivity better in concentrated than in dilute suspension, but this warning is frequently overlooked.

The loss of sensitivity of the red cells on ageing is clearly of importance when preparing the "pilot" bottle which is attached to the main bottle of blood for transfusion. The cells in the pilot bottle are used for cross-matching and sometimes for grouping, and if the red cells are in dilute suspension—a common practice—loss of sensitivity may be so great after some days' storage as to give a fa'se negative reaction in a cross-matching or grouping test.

In anthropological blood grouping, when many days may elapse between the taking of the blood in the field and its testing in the laboratory, care must also be taken to avoid loss of sensitivity of the red cells.

Preliminary work had indicated that the D antigen of the Rh system loses its sensitivity to agglutination fairly rapidly if red cells are stored in a weak suspension, and it is the purpose of this paper to present evidence of this phenomenon, as well as the slower loss of agglutinability of the A and B antigens on storage of blood under similar conditions. In contrast, it will be shown that this loss of sensitivity is greatly reduced if the concentration of the cells is increased by adding a large amount of blood to a small amount of anticoagulant.

Another possible source of error in the technique of cross-matching is contamination of the blood in the pilot bottle with a bacterium giving rise to panagglutination of the red cells. It was thought that contamination could be guarded against by adding penicillin and streptomycin to the anticoagulant in the pilot bottle. There is no evidence that addition of the antibiotics interferes with the action of Rh or ABO agglutinins on red cells.

Materials.

(a) *Anticoagulants.*

The anticoagulant reagents used were acid citrate dextrose (A.C.D.) solution, modified Rous-Turner (R.T.) solution, A.C.D. solution containing penicillin and streptomycin (A.C.D.P.S.), and a mixture of sodium citrate, penicillin and streptomycin in the solid form (C.P.S.).

(i) A.C.D. solution. This was prepared by dissolving 0·36 gm. of citric acid, 3·8 gm. of sodium citrate $2H_2O$, and 3·7 gm. of dextrose in 100 ml. of distilled water. The solution was sterilized by autoclaving.

(ii) Modified R.T. solution. This was prepared by the method described by Simmons and Graydon (1945).

(iii) Modified A.C.D. solution. This was prepared by dissolving 19 mg. of penicillin and 75 mg. of streptomycin in 22·5 ml. of the A.C.D. solution described above, and sterilizing by Seitz filtration.

(iv) Mixture of sodium citrate, penicillin and streptomycin in the solid form. 750 mg. of sodium citrate, 19 mg. of penicillin, and 75 mg. of streptomycin were weighed out and mixed. Approximately 85 mg. of the mixture were placed in each of a number of dry tubes and 15 ml. of blood were subsequently added to each tube and mixed.

(b) *Red cells.*

Blood was obtained by venepuncture and added to the four anticoagulants described above.

The amount of blood added was such that the concentration of blood in the A.C.D. solution and the R.T. solution was 5 p.c., in the modified A.C.D. solution 85 p.c., and in the solid anticoagulant 100 p.c. It is to be noted that Simmons and Graydon advocated an 8 p.c. concentration of blood in the R.T. solution, while Rous and Turner (1916) specified a 33 p.c. concentration.

The blood used was obtained from one group A Rh positive, one group A Rh negative, five group B Rh positive, one group B Rh negative, and three group O Rh positive donors. The blood from each of these donors was added to the four anticoagulants. To avoid bacterial contamination each of the four specimens was divided into six parts, one part being tested every three days and then discarded. All bloods were stored in the refrigerator until required for testing.

(c) *Anti-sera.*

The anti-A and anti-B sera were obtained from normal individuals of appropriate blood group, while the anti-Rh sera, both complete and incomplete, were obtained from women who had been immunized by the D antigen during pregnancy.

These sera were diluted serially at the beginning of the tests. The diluting fluid in the case of the complete antibody was saline, and in the case of the incomplete antibodies was AB serum, and the dilutions were kept frozen until they were required. This technique was adopted to avoid errors of dilution and deterioration of antibodies in weak dilution.

Method.

The only practicable method of determining the stability of the ABO and Rh antigens in the red cells at refrigerator temperature is to test the cells against decreasing quantities of the appropriate agglutinins at intervals of time after the withdrawal of the blood from the body. The end-point of the agglutination in the tests gives a measure of the stability of the antigens on storage.

The cells from all four preparations of blood were washed three times with normal saline solution and suspended in a 2 p.c. concentration. Where complete antibodies (anti-A, anti-B and anti-Rh) were being used, the cells were suspended in saline, and where the cells were tested against incomplete Rh antibody, they were suspended in 30 p.c. bovine albumin.

Equal quantities of the cell suspensions and the serially diluted sera were placed in agglutination tubes and incubated for one hour at 37° C.

After incubation the tubes were lightly centrifuged, the contents transferred to glass slides and examined for evidence of agglutination. In the case of incomplete antibodies it was found that sharper end-points were obtained if the tubes were shaken after centrifugation and the contents tipped out on to a glass slide for examination.

RESULTS.

It was thought that it would be clearer to portray the results in the form of graphs instead of presenting Tables showing the end-points of the various titrations. Since the titrations were carried out in the standard manner of doubling dilutions, the graphs are constructed logarithmically to the base 2.

Fig. 1 shows the results of titrating a complete anti-D serum against six D positive cells stored in the four different solutions after various intervals of time. The points of the curve represent the mean of the titration end-points of the various D positive cells stored under the same conditions. It will be seen that D positive cells stored as a weak suspension in A.C.D. solution become practically inagglutinable in two weeks' time, whereas stored as a strong suspension in A.C.D. solution, or with solid citrate, they are still agglutinable after

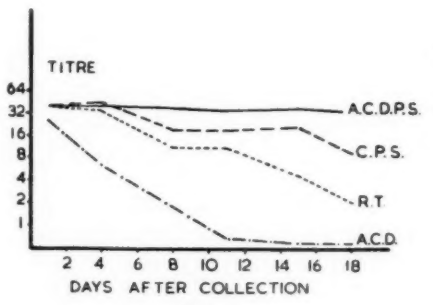

Fig. 1.

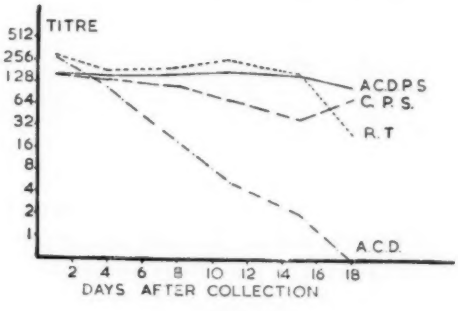

Fig. 2.

Fig. 1. Mean titres of complete anti-D serum tested against six Rh positive blood samples stored in different preservative solutions.

Abbreviations in this and following figures are:

A.C.D.=acid citrate dextrose solution.
R.T.=Rous-Turner solution.
A.C.D.P.S.=acid citrate dextrose solution with penicillin and streptomycin.
C.P.S.=citrate penicillin and streptomycin.

Fig. 2. Mean titres of incomplete anti-D serum tested against five Rh positive blood samples stored in different preservative solutions.

two weeks, although the titre has fallen. When stored as a weak suspension in modified Rous-Turner solution, the D antigen is more stable than when stored in A.C.D. solution in weak suspension.

Fig. 2 shows the results of titrating incomplete anti-D serum against five D positive cells stored under the same four conditions. The picture is very similar to that of Fig. 1. Again the D positive cells stored in weak suspension in A.C.D. solution soon begin to lose their sensitivity and in eighteen days' time have become inagglutinable by the incomplete anti-D serum.

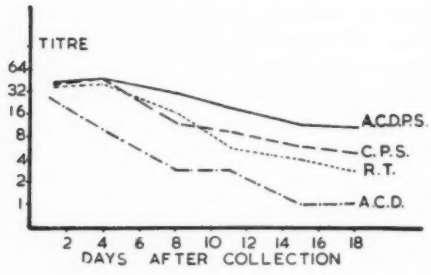

Fig. 3.

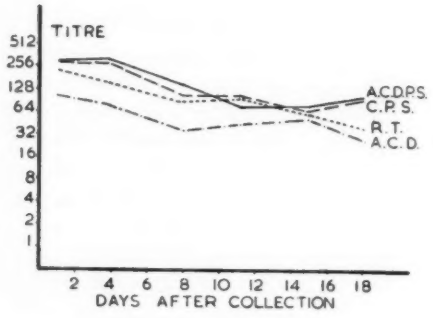

Fig. 4.

Fig. 3. Mean titres of anti-A serum tested against six group A blood samples stored in different preservative solutions.

Fig. 4. Mean titres of anti-B serum tested against six group B blood samples stored in different preservative solutions.

Fig. 3 shows the results of titrating an anti-A serum against six A cells stored in the four different ways at various intervals of time. It will be seen that there is a gradual decrease in sensitivity, but even when stored in weak suspension in A.C.D. solution, the loss of sensitivity of A cells is less marked than is the case with the D antigen.

Fig. 4 shows the result of a similar experiment with six B cells. Comparing Fig. 4 with Fig. 3, it might be thought that the B antigen is more stable than the A antigen, but this appearance is deceptive and is due to the greater strength of the anti-B serum. This is shown in Figs. 5 and 6, where the highest initial titres are called 100, and all other initial titres and subsequent titres are expressed in percentages of the highest initial titre.

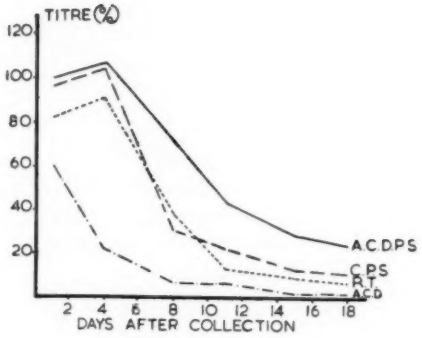

Fig. 5.

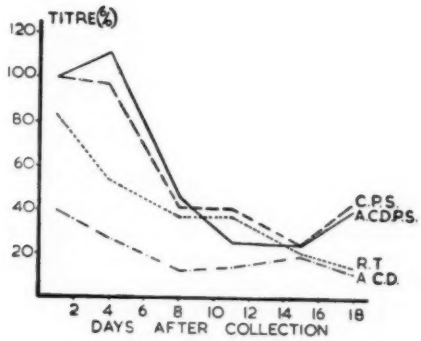

Fig. 6.

Fig. 5. Changes in titre of anti-A serum tested against six group A blood samples stored in different preservative solutions. (See text for method of calculation.)

Fig. 6. Changes in titre of anti-B serum tested against six group B blood samples stored in different preservative solutions. (See text for method of calculation.)

Fig. 5 shows the curves with the anti-A serum, Fig. 6 with the anti-B serum calculated on this percentage basis. It will be seen from Figs. 5 and 6 that there is no essential difference between the A and B antigens, the slope of the curves being approximately the same.

DISCUSSION.

In attempting to measure the progressive loss of sensitivity of the marker antigens of red cells during storage, the only practicable way is the indirect method of titrating the antibody and observing the end-point of the agglutination. Although indirect, it is reasonable to assume that it does give a measure of the sensitivity of the antigen. Is the antigen actually destroyed during storage or is it removed from the cells and passed into the suspending fluid? No attempts have been made to answer this question, but experiments on heated cells strongly suggest that the Rh antigens leave the cells and appear in the surrounding fluid.

In view of the experiments reported here, it is hardly necessary to stress the importance of storing red cells in strong suspension in storage fluids when they have to be kept for any length of time. This is of special importance in the case of the Rh antigen.

The Rous-Turner solution appears to be better than A.C.D. solution in preserving the antigens, but it would probably be better to adhere to the original proportions of blood and fluid, namely, 33 p.c. blood, advocated by Rous and Turner. This suggestion is supported by the recently published work of Nijen-

huis (1953), who found that the Rous-Turner solution, as modified by Simmons and Graydon (1945), was an excellent preserving fluid, but that the agglutinability of the red cells was definitely prolonged by increasing the amount of blood which was added to the fluid.

SUMMARY.

The agglutinability of the A, B and D antigens in human red cells on storage has been investigated, as it is often necessary, both in blood transfusion procedures and in anthropological investigations, to ascertain the antigens of red cells after varying periods of time.

Blood was added to four different preserving fluids and stored at 4–6° C. The blood was tested at three-day intervals up to 21 days. Preliminary work had indicated that the proportion of blood that was added to the preserving fluid was of importance, and special attention was paid to this point.

The agglutinability of all three red cell antigens investigated decreases on storage, whatever the preserving fluid, but the D antigen loses its sensitivity at a faster rate than the A and B antigens.

If the concentration of the blood in the preserving fluid is low, the agglutinability of the antigens decreases at a faster rate than if the concentration of blood is high. This phenomenon is again more marked in the case of the D antigen than with the A and B antigens.

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ON THE INTERACTION OF ^{32}P WITH AND ITS REMOVAL FROM DENATURED LIVER PROTEIN

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(Accepted for publication 6th April, 1954.)

The quantitative removal of inorganic ^{32}P from denatured protein precipitates by a simple washing process is essential, if it is intended that the specific activity (S.A.=counts/min./μg.) of organic P fractions in the protein residue be measured. Consequently, the difficulties reported by Davidson, Frazer and Hutchison (1951) in removing inorganic ^{32}P from protein precipitates are of interest. In a preliminary communication (Ennor and Rosenberg, 1952a) experimental results are quoted which are contrary to those of Davidson *et al.* (1951) and the present report is concerned with an extension of these results.

MATERIALS AND METHODS.

Animals.

Liver tissue from well-fed male guinea-pigs of about 400 gm. body weight was used throughout. In some cases livers from untreated animals were employed and in others livers from animals which had received an intraperitoneal injection of ^{32}P (about 100 μc./100 gm. body weight) as $NaH_2{}^{32}PO_4$ 2 hours prior to sacrifice.

Preparation of liver samples.

The method used for the preparation of the liver suspensions and the subsequent treatment of the protein residue was similar to that described by Davidson *et al.* (1951) up to the stage of the separation of the nucleic acids. For this latter purpose the method of Schmidt and Thannhauser (1945) was employed since, for the purpose of these experiments, a clear cut separation of the nucleic acids was not considered necessary. In our hands the alkaline digest of the extracted protein residue was clear after incubation at 37° C. overnight and no appreciable turbidity, apart from considerable stream double refraction, was visible. All such digests, however, were filtered before acidification and on no occasion was any radioactivity detected in the washed filter paper.

Trichloroacetic acid (T.C.A.) washes.

The method adopted for the initial denaturation of the liver suspensions with T.C.A. and for the subsequent washing of the denatured protein precipitate was as follows:

The suspension was cooled to 0° C. and sufficient cold 25 p.c. (w/v) T.C.A. added to bring the final concentration to 10 p.c. The resultant mixture was vigorously shaken and the supernatant removed after centrifugation at 0° C. All subsequent washes were carried out by the same method and with the shortest possible interval between each. At no time was the temperature allowed to rise above 1° C.

^{32}P.

All ^{32}P used was obtained from the Atomic Energy Research Establishment, Harwell, England, and was in the form of $NaH_2{}^{32}PO_4$. Prior to use, it was boiled for several hours with small amounts of concentrated HNO_3 and H_2SO_4 to ensure breakdown of traces of non-molybdate reacting ^{32}P. The HNO_3 was removed by prolonged heating, and a small amount of water added to make the resultant solution about N with respect to H_2SO_4. The solution was then heated at 100° C. for several hours to hydrolyse any pyrophosphate which may have been present (Ennor and Rosenberg, 1954). The hydrolysed material was then neutralized and used for injection, after the addition of a small amount of carrier P.

Determination of ^{31}P and ^{32}P.

^{31}P was determined as described by Ennor and Stocken (1950) and ^{32}P by the method of Ennor and Rosenberg (1952b). On occasions determination of the radioactivity was made directly on the T.C.A. washes and as a routine on the alkaline digests since the radioactivity was too low to permit dilution.

EXPERIMENTAL.

In the experiments to be described, inorganic ^{32}P has been recovered from:

(a) T.C.A. suspensions of the livers of non-treated animals; and
(b) from livers of animals which had received an injection of ^{32}P.

in both series the S.A.'s of inorganic P and certain organo P fractions have also been measured.

The recovery pattern of ^{32}P added as $NaH_2{}^{32}PO_4$ to T.C.A. suspensions of normal liver shows (Table 1) that over 99·8 p.c. of the total amount of ^{32}P recovered is present in the T.C.A. washes. The remainder was recovered in the lipid fraction and in the alkali soluble fraction. It will also be noted that excellent recoveries of added ^{32}P were obtained.

TABLE 1.

Distribution of ^{32}P added to "normal" liver homogenates as $NaH_2{}^{32}PO_4$ together with 25 p.c. trichloroacetic acid.

	Experiment 1		Experiment 2		Experiment 3	
	Counts/min.	P.c. of total recovery	Counts/min.	P.c. of total recovery	Counts/min.	P.c. of total recovery
Total ^{32}P added	1,123,850	—	582,000	—	1,310,500	—
^{32}P recovered as:						
Inorganic P	1,137,500	99·80	580,630	99·58	1,289,500	99·28
Acid sol. organic P	1,500	0·13	1,500	0·26	8,710	0·68
Total acid sol. P	1,139,000	99·93	582,130	99·84	1,298,210	99·96
Lipid-P	380	0·03	570	0·10	250	0·02
DNA-P	—	—	—	—	—	—
RNA-P	—	—	—	—	280	0·02
Phosphoprotein P	—	—	—	—	—	—
Total alkali sol. P.	410	0·04	390	0·07	280	0·02
Total recovered	1,139,790	100·00	583,090	100·00	1,298,740	100·00
P.c. of added P recovered	101·4	—	100·2	—	99·1	—

These initial experiments deal only with recovery of ^{32}P and take no account of S.A.'s which are the correct bases for comparison. Thus, the small amount of ^{32}P (<0·2 p.c. of the total recovered) which remains in the protein may lead to a false value for the S.A. of some organic P compound. Such a possibility has in fact been commented upon by Davidson *et al.* (1951). These authors have also noted that if ^{32}P as orthophosphate was added with the T.C.A. to a homogenate of a liver from an animal to which a prior injection of ^{32}P had been given, the S.A.'s of certain organic P compounds were increased appreciably due to what was considered by the authors as probably physical adsorption of ^{32}P onto the protein.

Some experiments similar to those on which this opinion was based have been carried out. Saline suspensions of livers from animals receiving a prior injection of ^{32}P were prepared. Each was divided into two equal portions and to one portion was added a sufficient volume of 25 p.c. (w/v) T.C.A. to bring the final concentration to 10 p.c. (w/v). A similar procedure was adopted with the remaining portion except that the T.C.A. added contained a known amount of ^{32}P as $NaH_2{}^{32}PO_4$.

TABLE 2.

Distribution of ^{32}P (counts/min.) added as $NaH_2{}^{32}PO_4$ together with T.C.A. to homogenates of livers from animals previously injected with ^{32}P.

Columns A include figures from the homogenates to which was added T.C.A. containing ^{32}P.
Columns B include figures from the homogenates to which T.C.A. only was added.

Liver fraction	Experiment 1 A ^{32}P added in TCA	Experiment 1 B control	Experiment 1 A–B	Experiment 2 A	Experiment 2 B	Experiment 2 A–B	Experiment 3 A	Experiment 3 B	Experiment 3 A–B
Ortho-P	181,200	104,625	76,575	660,075	227,950	432,125	916,950	179,365	737,585
Acid sol. organic P.	90,750	90,625	125	235,350	231,650	3,700	218,890	215,075	3,815
Lipid-P	19,240	19,250	0	33,950	34,220	0	A<B	—	0
RNA-P	—	—	—	—	—	—	12,150	11,075	1,075
DNA-P	—	—	—	—	—	—	900	900	0
Phosphoprotein P	—	—	—	—	—	—	A<B	—	0
Total alkali sol. P	9,880	9,200	680	22,120	21,800	320	—	—	—
Residue P	—	—	—	0	0	0	8	0	8

The protein precipitates were then given 8 washes with 10 p.c. (w/v) T.C.A. and the residue treated as before. The results (particularly columns A-B, Table 2) give a recovery pattern of the ^{32}P similar to that already shown in the preceding Table.

From the analysis of these results (Table 3) it is apparent that inorganic ^{32}P distributes itself in a constant manner irrespective of the presence or absence of ^{32}P in the organic P fraction. It would thus be anticipated that little change would occur in the S.A.'s of the organo P compounds, and this is borne out by the results (Table 4) of experiments in which both ^{31}P and ^{32}P have been measured.

TABLE 3.

Distribution of ^{32}P (p.c. of total recovery) added with T.C.A. to liver homogenates of guinea-pigs previously injected with ^{32}P as $NaH_2{}^{32}PO_4$.

	Experiment 1		Experiment 2		Experiment 3	
	counts/min.	p.c. of total ("B")	counts/min.	p.c. of total ("B")	counts/min.	p.c. of total ("B")
Actual ^{32}P added "A"	82,000	—	433,000	—	747,500	—
Recovered as:						
Inorganic P	76,575	98·96	432,120	99·08	737,585	99·34
Acid sol. organic P	125	0·16	3,700	0·85	3,815	0·51
Lipid-P	0	0	0	0	0	0
Alkali soluble P	680	0·88	320	0·07	1,075	0·14
Alkali insoluble residue	0	0	0	—	8	0
Total recovered ("B")	77,380	100·00	436,140	100·00	742,480	100·00
P.c. recovery ("B/A")	94·37	—	100·72	—	99·34	—

TABLE 4.

Effect of addition of ^{32}P as $NaH_2{}^{32}PO_4$ to denatured homogenates on the S.A.'s (counts/min./μg. P) of certain P-containing compounds in the tissues.

			Experiment 3**		Experiment 4**	
P group	Experiment 1*	Experiment 2*	^{32}P added	Control	^{32}P added	Control
Inorganic P	1,868	969	506	147·8	886·0	176·0
Acid sol. organic P	3·3	1·63	54·4	53·0	—	—
Lipid-P	0·17	0·04	5·69	5·53	6·1	6·9
RNA-P	0·42	0·1	4·23	4·96	3·8	3·55
DNA-P	0	0	1·31	1·07	0·81	0·64
Phosphoprotein P	—	0	51·5	40·60	34·3	36·2

* Experiments 1 and 2 were carried out with normal liver homogenates to which ^{32}P was added with the TCA.

** Experiments 3 and 4 were carried out with homogenates from livers of animals which had received an injection of ^{32}P 1 hour prior to sacrifice.

In these experiments the S.A.'s have been calculated both in the case of the control homogenates and those to which T.C.A. containing ^{32}P had been added. A comparison of the two results is of interest, and it will be seen (Table 4) that the addition of T.C.A. containing ^{32}P had little or no effect on the S.A.'s, as would be anticipated from the fact that nearly all the added ^{32}P can be recovered in the acid-soluble fraction. This fact is not in accord with the findings of Davidson *et al.* (1951), who have reported difficulty in washing out inorganic ^{32}P from T.C.A. precipitated liver tissues. In fact these authors found considerable amounts of ^{32}P in even the 47th wash of a normal liver to which T.C.A. containing ^{32}P had originally been added. The distribution of ^{32}P throughout successive washes of both normal livers, to which ^{32}P was added with the initial T.C.A., and of livers from animals to which ^{32}P had been administered prior to sacrifice show similar patterns (Table 5). In our hands the ^{31}P content of the

TABLE 5.

^{31}P and ^{32}P analyses on the T.C.A. washes of protein precipitates from homogenates of livers from normal guinea-pigs and those injected with ^{32}P as $NaH_2{}^{32}PO_4$ prior to sacrifice.
All figures refer to the total ^{31}P or ^{32}P in each wash (50 ml.).

	Experiment 1			Experiment 2			Experiment 3		
	^{31}P μg.	^{32}P counts/min.	S.A. counts/min./ μg. P	^{31}P μg.	^{32}P counts/min.	S.A. counts/min./ μg. P	^{31}P μg.	^{32}P counts/min.	S.A. counts/min./ μg. P
Original supernatant	—*	81,950	—	739	130,100	176·0	695·0	1,299,250	1,869
Wash 1	—	15,250	—	224	37,750	168·5	64·0	109,750	1,715
Wash 2	—	5,900	—	63·1	8·700	137·9	30·5	52,400	1,718
Wash 3	—	1,400	—	25·8	2,100	81·4	12·5**	10,700	856
Wash 4	—	125	—	19·1	600	—	5·0**	1,550	310
Wash 5	—	0	—	10·5**	115	—	3·0**	890	297
Wash 6	—	—	—	11·5**	0	—	2·0**	560	280
Wash 7	—	—	—	—	—	—	—	175	—
Wash 8	—	—	—	—	—	—	—	0	—
Wash 9	—	—	—	—	—	—	—	—	—

* Not estimated.
** Figures can no longer be considered as accurate, since only approximately 0·2 μg. or less per ml. was detected.

TABLE 6.

A comparison of the case with which ^{31}P and ^{32}P may be removed from different solid-liquid systems by washing and subsequent centrifugation.

System			Method of solute estimation	Unit	Conc. of solute in first supernatant (S) and subsequent washes (W)						
Solvent	Solid	Solute			S	W1	W2	W3	W4	W5	W6
0·5 p.c. NaCl in H_2O	Fuller's earth	^{32}P	Radioactive measurements	counts/min.	980	62	15	5	—	—	—
0·5 p.c. NaCl in H_2O	Finely precipitated silica gel.	^{32}P	Radioactive measurements	counts/min.	746	52	44	8	10	2	3
		^{31}P	Colorimetric measurements	μg. P	19·2	2·4	0·9	0·9	0·9	0·6	0·6
				S.A.	38·9	21·7	48·9	8·9	11·1	3·3	5·0
5 p.c. T.C.A.	Casein	^{32}P	Radioactive measurements	counts/min.	8,272	2,432	—	262	54	15	4·0*
		^{31}P	Colorimetric measurements	μg. P	293·5	88·6	—	10·4	2·2	0·9	0·4
				S.A.	28·2	27·4	—	25·2	24·5	16·7	10·0

* The casein precipitate after 6 washes was wet-ashed and the ^{32}P and ^{31}P estimated. Analysis showed a total of 185 μg. P and 7 counts/min.; hence S.A.=0·04.

3rd–4th T.C.A. wash fell below the level (1 μg. P/ml.) at which reliance could be placed upon the determinations. As far as ^{32}P was concerned, no radioactivity could be detected beyond the 8th wash, even when the ^{32}P added to the homogenate (50 ml.) was equivalent to $2{\cdot}5\times10^6$ counts/minute. The results are therefore in agreement with those of McCarter and Steljes (1948) and Dziewiatkowski and Bodian (1950).

In the third experiment (Table 5), where more data are available than in the other two, it will be noted that although ^{31}P could be detected in the 3rd-6th wash the amounts which were present were too small for accurate determination. Thus, in the 3rd wash, the 12·5 μg. P found corresponds to 1·25 μg. P in a 5·0 ml. sample which was the maximum volume which could be handled by the analytical method employed. This amount of P, and therefore the amounts quoted in the succeeding washes, falls below that which can accurately be estimated by the method and must therefore be regarded as erroneous, as indeed is shown by the fact that there is a progressive fall in the S.A.'s of the inorganic P recovered in each successive wash after the third.

The results indicate that since practically all the ^{32}P is recovered in the initial few washes, there is little possibility of adsorption of inorganic ^{32}P on the protein precipitate as has been suggested by Davidson *et al.* (1951) and by Marshak and Calvet (1949). As a test of this expression of opinion, ^{32}P has been recovered from a number of systems each consisting of an insoluble solid and a liquid phase. The results obtained (Table 6) are very similar to those obtained with liver samples, i.e., the bulk of the ^{32}P is recovered in four washes, and there is a progressive decline in the S.A. of the inorganic P after the third wash.

DISCUSSION.

The present results indicate that there is little, if any, ab- or adsorption of inorganic ^{32}P onto the protein and other T.C.A.-insoluble constituents of denatured liver. This conclusion is not in accord with that of Davidson *et al.* (1951), who were able to recover only about 60 p.c. of the added ^{32}P as inorganic P. It is difficult to explain this difference in findings unless it be due to differences in analytical techniques.

Another difference between the present results and those of Davidson *et al.* (1951) lies in the comparative ease with which inorganic ^{32}P may be removed from T.C.A. denatured homogenates of livers from animals which had received a prior injection of ^{32}P. Thus the Glasgow group were able to detect inorganic ^{32}P in all T.C.A. washes up to the 57th wash, whereas it was not possible in this work to detect any ^{32}P beyond the 5th–7th wash. This difference in results suggests that an explanation may be found in the techniques employed to wash the protein residues. Thus, if washing was at all prolonged, it might be expected

that ^{32}P would be split from the more labile organo P compounds in the protein residue. The fact that Davidson *et al.* (1951) have been able to detect ^{32}P even after numerous washes suggests that this may have arisen from such compounds. This conclusion is strengthened by the finding of Davidson and Smellie (1952). These workers, using an ionophoretic method for the separation of the components in the nucleic acid fraction, obtained in addition to RNA and DNA six other P-containing fractions, none of which could be identified with inorganic P.

The present results are of interest from the viewpoint of the reliability which can be placed upon calculations of S.A.'s. Thus it will be noted (Table 5) that there is a progressive drop in the S.A. of the inorganic P in the 2nd–6th washes. This drop is entirely due to the unreliability of the ^{31}P determinations at these low levels, as is proven by later experiments (Table 6) where similar decreases in S.A. are apparent even though ^{31}P and ^{32}P were recovered from completely inert material, in the original composition of which, except for casein, P was absent. The fall in S.A. values cannot therefore be due to the splitting off of small amounts of inert P from casein (Table 6) or from denatured liver tissue (Table 5). It is therefore important to remember that, in experiments involving the calculation of the S.A. of P in the inorganic or organic form, one is dependent upon the results of two measurements—one chemical and one physical—the sensitivities of which are greatly different. The ultimate reliability then of the calculation has as its limit the sensitivity of the chemical method.

From the present results it is not possible to suggest with certainty a source of the inorganic ^{32}P which Davidson *et al.* (1951) have found, but one possible source would seem to be the "phosphoprotein" fraction of the protein residue. The high S.A. of this fraction in the RNA and DNA, as separated by the Schmidt and Thannhauser (1945) procedure, leads to the belief (Jeener, 1949; Davidson *et al.*, 1951) that contamination with inorganic ^{32}P was present. If this is so, then the differences between the present work and that of Davidson *et al.* (1951) can be explained. In addition, it may be assumed that the relatively high value for the S.A. of phosphoprotein P indicates not a contamination but a higher turnover rate than either RNA or DNA.

Since this work was completed, similar conclusions have been reached by Johnson and Albert (1953), and it is hoped that work at present in progress in this laboratory will lead to a definite explanation of the differences mentioned and to an identification of the source of the inorganic ^{32}P in the experiments of Davidson *et al.* (1951).

SUMMARY.

The ease with which inorganic ^{32}P may be washed from protein precipitates with T.C.A. has been investigated.

It is concluded that inorganic ^{32}P may be removed from denatured liver protein with 5–8 washes of T.C.A.

The addition of inorganic ^{32}P to denatured liver protein containing ^{32}P in the organic P fraction leads to no change in the specific activity of the organic P.

Acknowledgments. We wish to thank Mr. R. Frater and Mr. D. Maguire for skilled technical assistance.

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AN ANALYSIS OF THE ADAPTATION OF AN INFLUENZA VIRUS TO PRODUCE LESIONS IN THE MOUSE LUNG

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(Accepted for publication 30th March, 1954.)

One of the classical instances of adaptation of a virus to a new host is the production of mouse virulent strains of influenza viruses. With some doubtful exceptions influenza viruses from natural human infections have no power whatever to produce lung lesions on intranasal inoculation of mice. If undiluted high titre allantoic fluid virus is used "toxic" lesions may be produced, but with a standard dose of 10 haemagglutinating doses no macroscopic changes are seen in the lung. With all influenza A viruses, however, some multiplication occurs and the virus persists on passage; eventually lesions appear and with further passage increase rapidly in intensity.

No attempt to analyse the nature of this process by genetic methods has been reported, although a good deal is on record in regard to the yield of virus at various stages of adaptation. Sugg (1949) used the influenza A prime strain, CAM, in a study of the "toxic" action of an unadapted virus in the mouse lung. He showed that with virus that had never been passed in mice the lesions produced by undiluted or low dilutions of allantoic fluid were proportional to the infectivity of the fluid as tested in the allantoic cavity. The lungs from the mice showing "toxic" lesions gave rise to no further lesions in the mouse lung for 2 further serial passages, although relatively high titres of virus were produced as shown by egg infectivity titrations. After 4 serial passages, however, lung lesions began to appear with a concomitant increase in the yield of mouse virulent virus.

Our objective has been to obtain evidence as to whether a series of mutational steps in the process could be detected and, by appropriate recombination experiments, to make a genetic analysis of pure clones at various stages of adaptation.

MATERIALS AND METHODS.

Virus.

The strain used throughout was the CAM strain of influenza A prime isolated by Anderson in 1946 (Burnet and Anderson, 1947) and maintained by infrequent amniotic or allantoic passages with intermediate storage in a dry ice cabinet. The stock virus used in these experi-

[1]This work was carried out under a grant from the National Health and Medical Research Council, Canberra.

ments was a frozen limit dilution allantoic fluid obtained after 7 amniotic and 6 allantoic passages. Two other lines were started to obtain mouse pathogenic and serum resistant stocks.

CAM antiserum.

Antiserum against CAM was obtained by immunization of two rabbits by intravenous inoculation of 5 ml. high titre allantoic fluid. The animals were bled 14 days later when the anti-haemagglutinin (AHA) of both sera were approximately 300, i.e., 1:300 dilution of serum just neutralized 5 agglutinating doses (AD) of virus. When CAM was titrated in the allantoic cavity a typical fluid gave ID_{50} of $10^{8 \cdot 0}$ when dilutions were made in normal serum saline (NSS), and $10^{4 \cdot 8}$ when dilutions were made in rabbit antiserum 1:25.

Characterization of virus clones.

As in other studies of influenza virus genetics, we followed the general technique of working as far as possible with pure clone fluids obtained by titrating all experimental material to limit infective dilution (LD) and using positive fluids from the highest infective dilution for characterization.

The characters studied as a routine were serology, as tested by anti-haemagglutinin tests with the sera described above, and pathogenicity for the mouse lung. All routine tests of mouse lung pathogenicity (MLP) were made by inoculating groups of 4, 4-week old mice intranasally with 0·05 ml. of virus diluted to a titre of 1 AD. Since the agglutinating dose of virus is in terms of a volume of 0·25 ml., in our technique the amount actually inoculated is 0·2 AD, but throughout this paper it is more convenient to refer to any test being made with virus diluted to a titre of 1 AD as being made with 1 AD of virus. Mice were killed 7 days after inoculation and the lung lesions recorded conventionally, 1–3·9 for living mice according to the degree of consolidation, death on the 5th day or earlier as 5·0, on the 6th or 7th day as 4·0.

Whenever a result that would indicate any recombination of characters was observed, or a strain was needed for experimental work, the LD fluid was again titrated and several further LD fluids tested for the character in question.

Development of mouse pathogenic (MP) virus.

For mouse passage 8, 4-week old mice were inoculated intranasally with 0·05 ml. stock allantoic fluid virus diluted to 20 AD. Two days later 4 of the mice were killed and bronchial washings obtained by a technique similar to that used by Fazekas (1948). Briefly, the trachea and lungs were dissected out and a suitably mounted cannula tied into the laryngeal end of the trachea. Two aliquots of about 0·5 ml. saline were drawn into the lung by negative pressure and returned by positive pressure into the wider part of the cannula. The fluids were highly turbid with cells and were spun before passage. The bronchial washings from either 2 or 3 mice were pooled, and, until a haemagglutinin titre (HA) could be obtained, this fluid was used undiluted as the inoculum for the next series of mice. The remainder of each batch of mice was killed 7 days after inoculation and the lung lesions recorded. Lung lesions began to appear at the 8th passage; thereafter there was a rapid increase in the intensity of the lesions and there were no survivors among the mice inoculated with 1 AD from 17th to the 20th passages. Detectable amounts of haemagglutinin appeared in the bronchial washings from the 12th passage onwards. The washings were then diluted to 1 AD and 0·05 ml. used for the next passage. Table 1 gives an abbreviated summary of the passage series.

TABLE 1.

Development of mouse pathogenicity of CAM by mouse lung passage.

Passage No.	H.A. titre of 2-day bronchial washings	Allantoic infectivity ID_{50}	Lesions in mice	Mean
4	<1	$10^{5.0}$	0, 0, 0, 0	0·0
8	<1	$10^{5.5}$	0, 0·5, 0·5, 2·0	0·75
12	1±	$10^{5.9}$	4, 4, 4, 1·5	3·4
16	6	$10^{6.1}$	4, 4, 4, 3·7	3·9
20	2	$10^{6.0}$	4, 4, 4,	4·0

The scoring convention for mouse lung lesions is described under Methods.

All bronchial washings were ampouled and stored in a dry ice refrigerator for subsequent study. The passage series was terminated after 20 passages.

A high titre antiserum, F387, against the CAM-MP strain was produced by the intranasal installation of allantoic fluid containing 20th mouse passage virus into a ferret which was bled out 10 days later. The characteristics of this antiserum are described in a later section (*vide* Table 3).

Development of serum-resistant (SR) virus.

Adaptation of the stock CAM strain to resist CAM antiserum was begun by allantoic titration of low dilutions of virus prepared in undiluted antiserum. The highest positive result was in 10^{-1} dilution. This fluid was used similarly and passage was continued as shown in Table 2.

TABLE 2.

Development of serum resistance of CAM by allantoic passage in anti-CAM serum.

Passage No.	Inoculum	H.A. titre of inoculum	Infective titre ID_{50} in anti-serum (dilution shown)		No. positive	Serial No. of representative fluid
1	10348	1000	AS/5	$10^{3.0}$	3/4	12768
2	12768	1200	AS/1	$10^{1.0}$	1/4	12974
3	12974	80	AS/1	$10^{1.0}$	2/4	13268
4	13268	80	AS/1	$10^{4.0}$	1/4	14392
5	14392	35	AS/3	$10^{3.0}$	1/4	15799
			nil*	$10^{5.0}$		
6	15799	40	AS/3	$10^{5.0}$	1/4	15871
7	15871	35	AS/3	$10^{4.0}$	2/4	16210
			nil*	$10^{5.0}$		
8	16210	500	nil*	$10^{5.0}$	2/4	16434

* No anti-serum used. Dilutions prepared in N.S.S.

The characteristics of the strain CAM-SR, represented by LD fluid 16434, were as follows: HA titre 300, infectivity in the allantoic cavity $10^{5.0}$ when diluted either in normal serum-saline or immune serum 1:25. In AHA tests 5 AD's were not neutralized by anti-CAM serum R853 1:50. With an anti-CAM-MP ferret serum, F387, which gave a titre of 3,000 against normal CAM, CAM-SR fluids gave values between 60 and 250. The strain had no patho-

genicity for mice when 1 and 10 AD doses were inoculated intranasally. With 100 AD's the results were irregular, but some mice died and others showed varying degrees of lung consolidation. This effect may well have been toxic in nature.

Antisera against the CAM-SR strain were produced in rabbits in the same way as anti-CAM serum (see above), except that the rabbits received a second subcutaneous injection of antigen emulsified with a paraffin and lanoline base 10 days after the initial intravenous inoculation. They were bled 14 days after the second injection. Only one serum, R863, was of satisfactory titre. The immunological aspects of the CAM-SR strain will be discussed in a separate paper. The relevant characteristics of the CAM-MP and CAM-SR strains are shown in Table 3 together with those of the original strain.

TABLE 3.

Characteristics of the two modified strains of CAM.

Virus	Usual H.A. titre of allantoic fluid virus*	M.L.P.†	Reduction of allantoic ID_{50} by CAM anti-serum 1/25	Antisera‡ R853 (CAM)	F387 (CAM-MP)	R863 (CAM-SR)
CAM	300–1000	>10	10^4	300	3000	2000
CAM-SR	80–300	>10	nil	50	150	3000
CAM-MP	300–1200	0·001	10^4	300	3000	2000

* Virus grown under the standard conditions of this laboratory.

† M.L.P.: Mouse lung pathogenicity as indicated by the AD's necessary to produce consolidation of approximately half the lung.

‡ Antisera: A.H.A. titres shown against 5 AD's of virus.

EXPERIMENTAL.

The preliminary work that has been reported under "Materials and Methods" provided three strains of virus. Stock CAM, serum sensitive and non-pathogenic for mice, and its two derivatives, MP, isolated after 20 mouse passages with high MLP and fully sensitive to anti-CAM serum, and SR, serologically easily distinguishable by its high resistance to anti-CAM serum and with no MLP. In addition, bronchial washings from mice representing virus at all stages of adaptation to the mouse lung were available. The main topics for investigation were (1) the process by which MLP was developed, and (2) an attempt to analyse recombination between MP and SR viruses. In the first section attention was mainly devoted to the stage at which mouse lung lesions first appeared, i.e., the 8th and 9th passages.

Analysis of 8th passage bronchial washings.

Two titrations of 8th passage bronchial washings and one of 9th were made in eggs and 11 and 4 pure clone fluids, respectively, were tested for MLP at the standard level of 1 AD. The average value of the lung lesions observed in 4 mice inoculated with each fluid were:

8th—0, 0, 0, 0, 0, 0, 0, 0·1, 0·3, 0·4, 0·65.
9th—0, 0·15, 0·25, 0·9.

Further LD fluids were obtained by titrating some of these fluids in the allantoic cavity. These were also tested in mice with the results shown in Table 4.

TABLE 4.

Mouse lung pathogenicity of re-isolated clones from 8th mouse passage of CAM.

1st LD series		2nd LD series		
Allantoic fluid No	Mouse lung lesions* 1 AD dose	Allantoic fluid No.	Mouse lung lesions 10 AD dose	1 AD dose
16644	0·0	18386	0·1	
16645	0·4	19509	0·9	0·8
		19511	0·4	0·7
		19512	0·2	0·1
16648	0·0	18339	0·0	
		18340	0·0	
		18342	0·1	
16649	0·3	19522	2·1	0·4
		19526	0·8	0·3
		19527	1·0	0·15
14249	0·1	15642		0·2
		15643		0·45
16651	0·65	18367	0·0	
		18372	0·0	
		18373	0·0	

* The scoring convention for mouse lung lesions is described under Methods.

These results show first that the 8th passage material contains virus particles of at least 2 grades of virulence, one (represented by 16644 and 16648) which gives no lesions with 1 AD doses and at most a hardly significant 0·1 lesion with 10 AD. The second is represented by 16645 and 14249 in which small but definite lesions are produced by 1 AD doses with maintenance of this character on LD passage. Fluid 16651 is anomalous and presumably represents a mixture of the two types, the virulent being overgrown by the non-virulent, so that it was not present in the 2nd LD generation.

The 8th passage LD fluids tested gave 7/11 with no pathogenicity at the 1 AD dose level while the 9th gave only 1/4.

In the 12th and 16th mouse lung passages, LD fluids from the first allantoic titration of the bronchial washings were tested for mouse lung pathogenicity. Three fluids from the LD titration of the 12th mouse passage virus were given a further egg passage at low dilution and were also tested in mice. The combined results are shown in Table 5.

TABLE 5.

Mouse lung pathogenicity of re-isolated clones from the 12th and 16th mouse passage of CAM.

		Average mouse lung lesion scores						
12th M.P.	1st allantoic passage (LD fluids)	1 AD dose						
		0·1		2·1		3·0	3·3	3·6
		10 AD	1 AD	10 AD	1 AD		10 AD 1 AD	
	2nd allantoic passage	0	0	0·2	0·27	not done	2·3 1·7	not done
16th M.P.	1st allantoic passage (LD fluids)	1 AD dose						
		3·5	3·9	4·0	4·0	4·0	4·4	4·5

The results given in Table 6 indicate that at least from the 8th passage onward there is a progressive change in the composition of the virus population obtained from the bronchial washings.

TABLE 6.

Change of virus population with passage. Distribution of LD fluids based on M.L.P.

Mouse Passage No.	Group 0 M.L.P. score 0	I 0·1–0·5	II 0·6–1·5	III 1·6–3·9	IV 4·0+
8	7	3	1	0	0
9	1	2	1	0	0
12	0	1	0	3	0
16	0	0	0	2	5
20	0	0	0	0	6

Changes which can be represented by group numbers, O, I, II, III, IV, occur, the changes being such that IV has a selective survival advantage over III, which has the advantage over II, and so on. These changes are inheritable but there seems to be a possibility that some or all of the progeny, on LD passage in the allantoic cavity, will slip back one place in the series. It is not possible on these findings to say whether the process is the result of a series of mutations or due, as we have suggested in somewhat similar situations, to an increasing development of labile "virulence" genes.

Recombination experiments between CAM-MP and CAM-SR.

These experiments followed the standard pattern developed with the deembryonated egg technique (Burnet and Lind, 1954a), 5 ml. of fluid containing a mixture of viruses, at a final titre of 10–20 AD of each virus, being used as

inoculum. After one hour's incubation to allow for virus absorption, the eggs were treated with RDE for an hour then washed thoroughly, refilled and returned to the incubator. Fluids containing the products of the first growth cycle (first cycle fluids) were harvested 7 hours after infection and stored in a dry ice box.

The first cycle fluids were given a preliminary titration in the allantoic cavity and, on the basis of the results obtained, a second titration was made to provide a large number of LD fluids.

Table 7 shows the character of 18 LD fluids obtained from two titrations of first cycle fluid from the first experiment in which CAM-MP and CAM-SR were used as parent viruses.

TABLE 7.

Character of LD fluids from titration of first cycle fluid from first CAM-MP/CAM-SR recombination experiment.

	M.L.P. with 1 AD test dose		
Serology	Non mouse-pathogenic (No.)	Mouse pathogenic (average lesions shown for each fluid)	Total
Serum sensitive	2[1,2]	1·3[3] 1·5[4] 2·7[5] 4·4	6
Intermediate	1[6]	0·1[7] 0·2[8]	3
Serum resistant	14	0·15[9]	15

Index figures refer to virus fluids also shown in Table 8.

The features of interest are (1) the serum resistant virus has in 14/15 instances shown no change from its initial absence of MLP; (2) the serum sensitive character is now associated with a wide scatter in the values of MLP, two having no pathogenicity and one giving the standard degree of pathogenicity.

TABLE 8.

Retitrations of fluids from first SR/MP recombination experiment.

	M.L.P. with 1 AD test dose			
Reference*	Original	1st LD fluids	2nd LD fluids	Notes
1 (16596)	0·0	0·0, 0·0		
2 (16616)	0·0	0·0, 0·0	0·0	
3 (16470)	1·3	0·1, 0·2, 0·4		
4 (17031)	1·5	0·8, 0·9		
5 (16469)	2·7	2·5, 3·7, 3·9		
6 (16444)	0·0	0·0, 0·0, 0·1	0·0	This gave both SR and non SR types
7 (16598)	0·1	0·0, 0·3, 0·35		
8 (16603)	0·2	0·0, 0·35	0·0, 0·0	
9 (16614)	0·15	0·0, 0·0		Original a mixture (SR+MP)

* See Table 7.

Retitration in the allantoic cavity and characterization of LD fluids gave the results shown in Table 8. Only two of the fluids gave evidence of being mixtures, one of these was the only strain which appeared to combine serum resistance with mouse pathogenicity 9 (16614). The isolated SR component was, however, quite non-pathogenic. The other strains either corresponded in MLP or showed a variable fall. There were no significant increases in pathogenicity.

In the second experiment, in addition to CAM-MP/CAM-SR mixtures, two other series of de-embryonated eggs (DEE) received CAM-MP or CAM-SR alone. The results are summarized in Tables 9 and 10.

TABLE 9.

Character of LD fluids from allantoic titration of first cycle fluid from second CAM-MP/CAM-SR recombination experiment.

	M.L.P. (1 AD test dose of LD fluids)	
Inoculum	Serum sensitive	Serum resistant
MP-SR	0·1[1], 0·3[3], 2·8	0·0, 0·0, 0·0, 0·0, 0·0, 3·0[3]
MP alone	4·0, 4·3, 4·3, 5·0	
SR alone		0·0, 0·0

TABLE 10.

Retitrations of fluids from second MP/SR recombination experiment.

	M.L.P.			
Reference*	Original	1st LD fluid	2nd LD fluid	Notes
1 (17982)	0·1	0·1	(3·0) (1·1)	SR isolated from this fluid
2 (17969)	0·3	(1·5) (1·2)		
3 (17977)	3·0	0·4	(0·0) (0·15)	

Bracketed figures tests made with 10 AD virus, remainder with 1 AD.
* See index figures, Table 9.

The two parts of this experiment are consistent and the over-all results may be depicted, in the form used previously for similar studies, in Fig. 1.

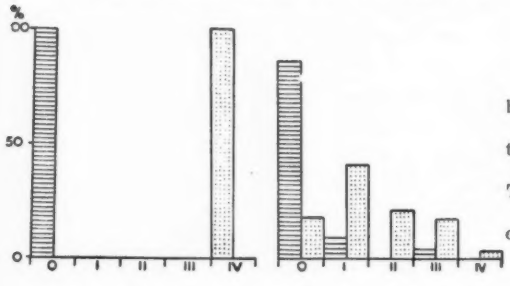

Fig. 1. Redistribution of mouse virulence in recombination between CAM-MP and CAM-SR.

Ordinates: Percentage of pure clone fluids falling in the grade shown for mouse lung pathogenicity.

Abscissae: Grades of mouse lung pathogenicity as in Table 6.

Fluids are grouped according to serological character, cross hatched—serum resistant: stippled—serum sensitive.

The Figure includes all fluids tested including those of LD passages from the primary isolates. No significant difference from what would be obtained with primary isolates alone is introduced by this procedure. It will be seen that there has been a marked redistribution of virulence amongst the serum sensitive offspring while the great majority of the serum resistant progeny still show no mouse pathogenicity whatever.

DISCUSSION.

This is the first paper of what is hoped will be a number concerned with the process by which a virus develops virulence by repeated passage in a new host. An influenza virus well adapted to the allantoic cavity, but never previously having been used to infect mice, is particularly suitable for this purpose. It can be titrated readily in the chick embryo and by haemagglutination so that one can distinguish between qualitative increase in virulence and simple increase in the amount of virus harvested. All influenza A strains rapidly develop virulence for mice which can be raised to a high level by uninterrupted 2-day passage. We have found the technique of passage by bronchial washings very satisfactory. It can be regarded as more "physiological" than grinding and extracting the lungs and it is easy to check the HA titre of the washing and so ensure passage with the standard 1 AD dose.

The results obtained are in accord with the hypothesis of the successive replacement of one population by another better able to multiply in the new environment, but it is not evident whether this process is one of successive mutations or of one or a very small number of mutations followed by overactive replications of the mutated "genes."

As we have found in other circumstances (Lind and Burnet, 1954), LD titration and testing of recently developed clones often shows a drift downward in virulence from that of the primary clone. This naturally speaks much more strongly for the second hypothesis since otherwise back mutations would have to be postulated as occurring with undue frequency.

The original intention of the work was to define first step mutations toward mouse virulence and use these in recombination experiments. It became obvious, however, that the lesions produced at the presumptive first step were minimal and that it would be quite impossible to study them without prohibitively large numbers of animals. The recombination studies were therefore made in the first instance with 20th mouse passage material of high virulence. It was regarded as highly desirable that recombination should be with an avirulent strain carrying an independent marker, and for this purpose the CAM-SR strain was developed.

The recombination experiments were expected to provide easily recognisable serum resistant mouse pathogenic strains, but in fact only one unstable example

of this type was obtained. On the other hand, the non-SR clones obtained from the experiment showed the expected redistribution of grades of virulence over the whole range. This was not shown when CAM-MP alone was subjected to the same series of manipulations.

It seems, therefore, that in the CAM-MP/CAM-SR cross the CAM-SR genome can divert virulence from CAM-MP multiplying in the same host cell, but is only most exceptionally capable of "accepting" virulence. This appears to be a similar phenomenon to that already encountered in the inability of MEL to accept virulence units for haemorrhagic lesions in the embryo (Burnet and Lind, 1954b).

The MP-CAM strain is also being studied in regard to other types of manipulation that will reduce its capacity to produce mouse lung lesions in the hope of throwing light on the difference between unstable and stable levels of virulence. For the present, however, the evidence is clear that double infection with a non-virulent strain can in one way or another greatly reduce the virulence of the strain and that this reduction in virulence is inheritable.

SUMMARY.

Two modified lines of the influenza A prime virus, CAM, were developed: (1) CAM-SR resistant to anti-CAM serum, and (2) CAM-MP fully adapted to growth in the mouse lung.

A study was made of the distribution of mouse lung pathogenicity among pure clone samples of the CAM-MP strain at different stages in the course of its adaptation to mice.

Recombination experiments between CAM-MP and CAM-SR yielded only one mouse pathogenic CAM-SR strain, and gave CAM-MP strains which showed a wide variation in their power to produce mouse lung lesions. Few of these latter strains had the lesion producing power of the standard CAM-MP strain.

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THE FLOW OF BLOOD IN NARROW TUBES

I. A CAPILLARY MICROPHOTOMETER: AN APPARATUS FOR MEASURING THE OPTICAL DENSITY OF FLOWING BLOOD

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(Accepted for publication 25th February, 1954.)

One of the most important factors causing the anomalous viscosity of blood in narrow tubes (see Bayliss, 1952) is the formation of a cell-free marginal zone. The degree of packing and orientation of the cells doubtless is also significant. In order to investigate these changes in a stream of blood flowing in a narrow capillary, an apparatus has been constructed which enables measurement of changes in the optical density of the stream at various points (stations) across a diameter.

This paper describes the construction of the apparatus and the method of operation, together with an assessment of its reliability.

METHODS.

Materials.

Fresh, defibrinated sheep's blood was used for these calibration experiments. Human blood was not used, for a reason dealt with below. Dilution, where required, was by serum collected from the same specimen by centrifugation; or by isotonic saline in those cases where the cells were suspended in saline after washing three times by centrifugation.

Haematocrit values were estimated by centrifuging the sample for one hour at 5,000 r.p.m. in a bucket centrifuge. The tubes were 5 cm. long and 1 mm. in bore.

Haemolyzed specimens were obtained by repeated freezing at −20° C. and thawing at room temperature. Cell debris was removed by centrifugation. This method gave nearly complete haemolysis.

Apparatus.

This consisted of a reservoir, from which a capillary tube passed under a microscope. The image of the tube was projected by a rotatable mirror, mounted just above the ocular, onto a fixed slit, behind which was a photo-multiplier tube. The projection distance varied with tubes of different bore, but was 40 cm. in the experiments reported here. The apparatus was not heat-controlled. The general disposition of the apparatus is indicated in Fig. 1.

The reservoir was made of perspex sheet and contained a well for the fluid. A sectional view of this is shown in Fig. 2. A needle-valve, at the same level as the capillary tube, allowed samples to be withdrawn or diluent added. The capillary tube was sealed into the well by a

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screwed gland packed with heavy grease. This usually blocked the entrance of the tube as it was being inserted. A few millimetres were therefore always broken off before the apparatus was used after fitting a new tube. The lid of the reservoir was held by six wing-nuts, a rubber gasket providing an air-tight seal. Mounted on top of the lid was a small turbine stirrer driven by compressed air. Its agitation was not observed to cause haemolysis. The reservoir was mounted on a rigid stand to bring the capillary tube to the level of the microscope stage.

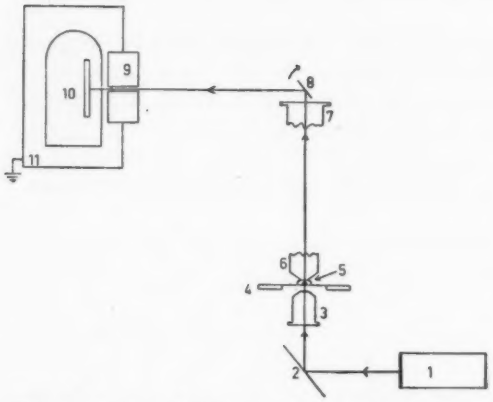

Fig. 1. General disposition of the apparatus. The capillary tube runs perpendicular to the plane of the page.

(1) Monochromator, vertical exit-slit.
(2) Front-aluminized mirror.
(3) 2/3 in. objective employed as condenser.
(4) Mounting for capillary tube.
(5) Oil-immersed capillary tube.
(6) 1/7 in. apochromatic oil-immersed objective.
(7) ×6 ocular.
(8) Rotatable scanning mirror, front-aluminized.
(9) Slit.
(10) Photomultiplier tube.
(11) Earthed copper housing.

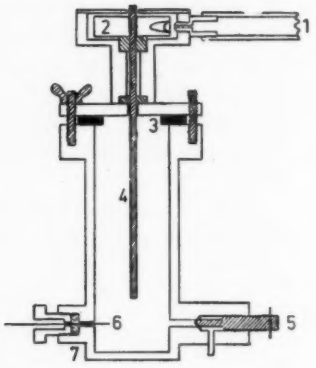

Fig. 2. Sectional view of reservoir.

(1) Compressed air line.
(2) Rotor.
(3) Rubber gasket.
(4) Stirrer shaft.
(5) Needle-valve.
(6) Capillary tube.
(7) Grease gland.

The capillary tubes were made by drawing out ordinary soda-glass tubing, 6–8 mm. in external diameter. The usual length obtained was about five feet. To select a suitable piece from this, a small globule of mercury was introduced into one end and moved along by compressed air. The length of the resulting thread was measured every 10 cm., and those portions selected where this remained constant. Only about one tube in three provided such a piece. The diameter of the tube was estimated by weighing it while containing a mercury thread of known length and re-weighing empty. This was done on a torsion balance reading to 1 mg. Checking of the calculated diameters by a microscope and graticule gave approximately the same results. Tubes were 15 cm. in average length and their diameters ranged from 0·350 mm. to 0·070 mm. It was not possible to flare the ends of these tubes.

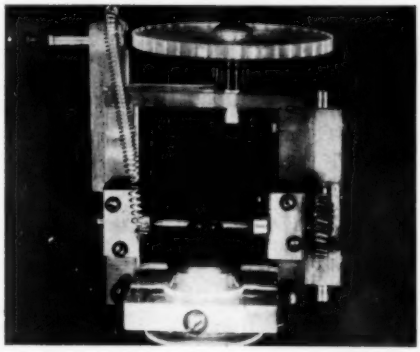

Fig. 3. Scanning mirror and mounting, details in text.

The microscope was a standard monocular model with a mechanical stage. The capillary was secured to a thin glass slide by strips of adhesive cellulose tape. The distance from the origin of the tube to the point of observation was approximately 10 cm. Light from a ribbon-filament tungsten lamp (6 v. 110 w.) was rendered monochromatic at 5,300 mμ by a Hilger quartz monochromator, and passed to the microscope condenser by a front-aluminized coverslip cemented to the substage mirror. A 2/3-in. objective was used as a condenser. A 1/7-in. oil-immersed apochromatic objective was used with a ×6 ocular. The exit slit of the monochromator (at right angles to the axis of the tube) and the transverse diameter of the tube were brought to focus together.

The scanner (Fig. 3). The small, front-aluminized scanning mirror was mounted about 1 cm. above the ocular on a rotatable shaft. This was supported by a perspex framework attached to the draw-tube of the microscope by four aligning screws. It was spring-loaded to prevent backlash in the train of gears through which it was driven by the notched wheel on top of the framework. A spring-loaded catch engaged in the forty notches of the wheel so that the mirror could be turned through small equal angles and returned to any given point. Rotation of the mirror caused the reflected image of the capillary tube to pass across the fixed horizontal slit in front of the photomultiplier tube. The width of the exit slit of the monochromator was so adjusted that its image just filled this slit, to which it was perpendicular.

The photomultiplier tube mounting consisted of a bakelite front plate secured to two uprights on a stand so that lateral and vertical adjustments were possible. Fixed in this plate was a brass block with a horizontal slot 1 mm. high, 1·3 cm. wide, and 2·5 cm. deep, the walls painted black. The photomultiplier tube (RCA 1 P 21) was contained in a light-tight, earthed copper housing on a second sheet of bakelite sliding in runners behind the first. Also in this sheet was a narrow sighting tube, which was brought behind the slit when lining-up the optical path. When not in use the photomultiplier tube was always kept to one side to preserve its dark-adaptation.

Electrical system. Initial instabilities in the system were removed by shielding the photomultiplier leads and earthing all neighbouring metal components. Current for the 240 6·5 v. lamp transformer and the D.C. amplifier was supplied from a heavy-duty autotransformer voltage-regulator connected to the 240 v. A.C. mains. The photomultiplier was powered by ten 90 v. miniature radio dry batteries connected in series. Its output was amplified by the D.C. amplifier, readings being taken from a large-scale 0–20 μa. moving-coil galvanometer.

Air pressure was controlled by admitting air to the reservoir from a cylinder through a regulating valve. A side tube on the air line was connected to a U-tube mercury manometer. Negative pressure, when required, was provided by a filter-pump.

Techniques.

Measurements of the optical density of flowing blood were made as follows. The sample of blood was placed in the reservoir, the lid screwed down and the stirrer set in motion. The room was darkened, the photomultiplier slid into position behind the slit, and the mirror rotated until the image of the edge of the capillary was brought to the slit. This could be determined by a sudden fall in the galvanometer reading. With a card in front of the exit slit of the monochromator, the dark-current control was then adjusted to bring the galvanometer reading back to zero, thus compensating for stray light. This adjustment was checked periodically in the course of observations. After an initial period of about 15 minutes, the apparatus was warmed-up and stable, and little adjustment was required. The sensitivity of the amplifier and the intensity of the light were then adjusted to give a workable reading.

In the calibration experiments reported in this paper, a second method of filling the tube with fluid was employed. The samples of whole blood, haemolyzed blood, saline or serum were contained in 1 ml. pipettes arranged horizontally, side by side, so that their tips could be placed in turn against the end of the capillary tube. Continuous suction (−10 mm. Hg.) applied to the reservoir drew the required fluid into the tube, and after allowing time for the previous contents to be replaced, a reading was then taken. The mirror was then advanced to the next station and the process repeated until the tube had been scanned. A preliminary run having disclosed how many stations were required for a complete traverse of the tube, measurements were sometimes carried out only to a point just beyond half way. The blood was kept from settling in the pipette by rotating it through a quarter turn three or four times a minute.

Calibration of the photomultiplier was carried out by successively cutting out segments of a cardboard disc rotated in front of the slit at high speed by an electric motor. This was repeated at low, moderate and high light intensities with discs of 20 and 36 segments. Although a 50 c.p.s. fluctuation in the intensity of the light source could be demonstrated by a cathode-ray oscilloscope, no stroboscopic artefacts were detected in the calibration.

RESULTS.

Fig. 4 and Table 1 give the results of the calibration experiments. The deviation from a linear response is shown to be small, and the readings obtained in other experiments were therefore used uncorrected. The variation in amplifier setting did not influence the response.

TABLE 1.

Calibration of photomultiplier.

Segments removed	Galvanometer readings A	B	C	D
0	0·0	0·0	0·0	0·0
1	2·0	2·5	4·0	2·5
2	4·0	4·5	9·0	5·0
3	6·5	7·0	13·0	(?) 9·0
4	8·5	10·0	18·0	10·0
5	11·0	12·5	23·0	12·5
6	13·5	15·5	28·0	15·5
7	16·0	18·0	33·0	18·5
8	19·0	21·0	38·0	21·5
9	21·0	24·0	43·0	24·0
10	24·5	27·0	48·5	27·0
11	27·5	29·5	53·5	30·0
12	30·5	31·5	59·0	32·5
13	33·0	35·0	64·5	35·0
14	35·0	38·0	69·0	37·5
15	38·0	40·5	74·5	40·5
16	41·0	43·5	80·0	43·0
17	44·0	46·5	85·0	46·0
18	46·5	49·5	90·0	49·5
19	50·0	52·5	95·0	52·0
20	53·0	55·0	100·0	56·0
21	56·5	57·5	—	59·0
22	59·0	61·0	—	62·0
23	62·0	63·0	—	65·0
24	65·0	66·0	—	68·0
25	69·0	69·0	—	71·0
26	71·5	71·5	—	74·0
27	74·5	74·5	—	76·5
28	77·0	77·0	—	79·0
29	79·0	80·0	—	81·5
30	82·0	82·5	—	84·0
31	85·0	85·0	—	87·0
32	88·0	88·0	—	90·0
33	91·0	91·0	—	92·5
34	94·0	94·0	—	95·0
35	97·0	96·5	—	97·5
36	100·0	99·0	—	100·0

Column	Light intensity
A and B	High
C	Medium
D	Low

Three suspensions of sheep red cells in serum were prepared with haematocrit values of 18 p.c., 10 p.c. and 5 p.c., and their optical densities measured across the tube, relative to serum. They were then haemolysed and the measurements repeated. The tube, in this case, was 0·150 mm. in diameter. All six graphs were found to have a similar shape, almost semi-circular. As an illustration Figs. 5 and 6 give the optical density curves for the 18 p.c. sample. The figures from which these curves were constructed are given in columns 2 and 3, respectively, of Table 2.

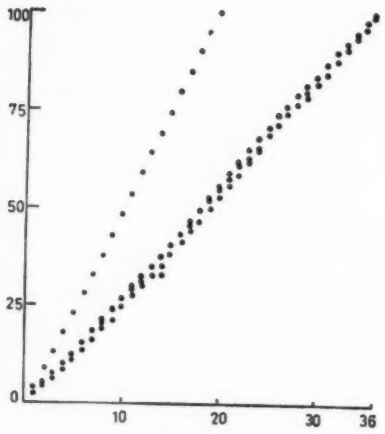

Fig. 4.

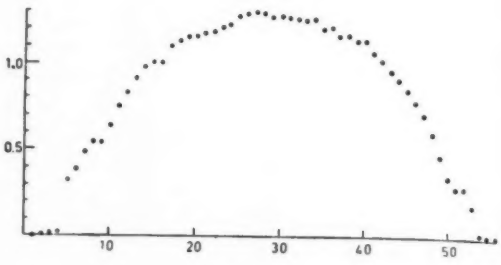

Fig. 5.

Fig. 4. Calibration curve of photomultiplier. Ordinate: galvanometer reading. Abscissa: Number of segments removed from disc. Solid circles: 36 segments employed. Open circles: 20 segments employed.

Fig. 5. Optical density of suspension of cells in serum. Haematocrit 18 p.c.
Ordinate: Optical density—relative to serum.
Abscissa: Stations of observation (edge of tube falls at 4).

In order to gain some knowledge of the optical properties of this system, the "effective optical path length" at the different stations was calculated from the densities of the haemolysed samples as follows: the mean of the three central values for the optical density, for each of the three samples, was divided into the optical density at each point. The quotient expresses the effective length of the optical path in terms of the diameter of the tube, it being 1·0 at the centre. Three sets of values for these lengths were thus obtained. Since the points of observation probably did not exactly coincide, the root-mean-squares of these values were calculated and appear in column 5 of Table 3. Values over half the tube are shown in Fig. 7. It may be seen that deviation from the circular arc is not great.

The effect of dividing an optical density by the length of the light path through the medium is to give the extinction coefficient, if the Lambert-Beer law is obeyed.

The "extinction coefficient," in arbitrary units, was found for the three suspensions of cells by employing the values for the effective length of the path found as described above. The values of this term for an 18 p.c. suspension are given in column 6 of Table 2. It was fairly constant except towards the edge of the tube. Some of this error was probably attributable to lack of coincidence of the points of observation, which, while having a negligible effect if occurring

TABLE 2.

Station	Optical density A Haemolyzed cells	Optical density B Intact cells	Ratio A/B	Length	Extinction coefficient B	Extinction coefficient A
1	0·1800	0·3322	0·54	0·24	1·38	0·75
2	0·2341	0·3819	0·61	0·37	1·03	0·63
3	0·2577	0·4831	0·53	0·36	1·34	0·72
4	0·3623	0·5402	0·67	0·56	0·96	0·65
5	0·3310	0·5380	0·62	0·44	1·22	0·75
6	0·3403	0·6426	0·53	0·50	1·29	0·68
7	0·4002	0·7533	0·53	0·59	1·28	0·68
8	0·4420	0·8331	0·53	0·64	1·30	0·69
9	0·4808	0·9008	0·53	0·70	1·29	0·69
10	0·5229	0·9769	0·49	0·75	1·30	0·70
11	0·5254	1·0508	0·50	0·78	1·36	0·67
12	0·5477	1·0740	0·51	0·81	1·33	0·68
13	0·5740	1·1004	0·52	0·85	1·29	0·68
14	0·5768	1·1326	0·51	0·87	1·30	0·66
15	0·5984	1·1566	0·52	0·90	1·28	0·66
16	0·6380	1·1601	0·55	0·93	1·25	0·69
17	0·6240	1·1833	0·53	0·94	1·26	0·66
18	0·6414	1·1880	0·54	0·94	1·26	0·68
19	0·6482	1·2111	0·54	0·97	1·25	0·67
20	0·6532	1·2340	0·53	0·99	1·25	0·66
21	0·6569	1·2765	0·51	0·98	1·30	0·67
22	0·6504	1·2981	0·50	0·99	1·30	0·66
23	0·6665	1·3078	0·51	1·00	1·31	0·67
24	0·6702	1·2987	0·52	1·00	1·30	0·67
25	0·6739	1·2787	0·53	1·00	1·28	0·67
26	0·6591	1·2833	0·51	1·00	1·28	0·66
27	0·6624	1·2765	0·52	0·99	1·28	0·67
28	0·6524	1·2695	0·51	0·99	1·28	0·66
29	0·6524	1·2624	0·52	0·98	1·30	0·67
30	0·6497	1·2648	0·51	0·99	1·28	0·66
31	0·6541	1·2066	0·54	0·97	1·24	0·67
32	0·6243	1·2139	0·51	0·95	1·28	0·66
33	0·6128	1·1737	0·52	0·92	1·28	0·67
34	0·5984	1·1712	0·51	0·93	1·26	0·64
35	0·5760	1·1461	0·50	0·88	1·30	0·65
36	0·5661	1·1437	0·49	0·86	1·33	0·66
37	0·5682	1·0792	0·53	0·82	1·32	0·69
38	0·5082	1·0248	0·50	0·81	1·27	0·63
39	0·5136	0·9668	0·53	0·74	1·31	0·69
40	0·4881	0·9345	0·52	0·72	1·30	0·68
41	0·4624	0·8531	0·54	0·68	1·25	0·68
42	0·4298	0·7809	0·55	0·66	1·18	0·65
43	0·3877	0·7079	0·55	0·58	1·22	0·67
44	0·3565	0·5988	0·60	0·51	1·17	0·70
45	0·3059	0·4658	0·66	0·47	0·99	0·65
46	0·2576	0·3499	0·74	0·40	0·87	0·64
47	0·2909	0·2814	1·03	0·41	0·69	0·69
48	0·1608	0·2872	0·56	0·28	1·03	0·57
49	0·1484	0·1814	0·82	0·28	0·65	0·53

at the centre of the tube, introduces large errors near the edge. This was exemplified by the application of the same process to the figures for optical density of the corresponding haemolyzed specimen, the "extinction coefficient" as calculated at the various stations of observation being given in column 7 of Table 2. Similar results were obtained for the other specimens.

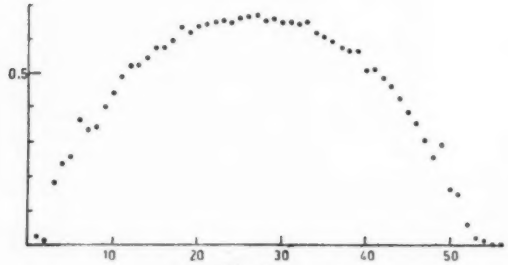

Fig. 6.

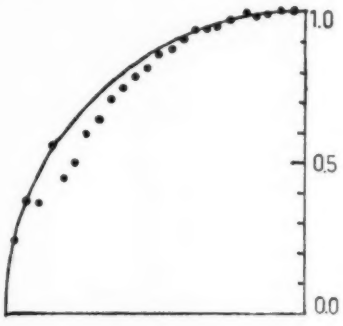

Fig. 7.

Fig. 6. Optical density of lysed cells in serum, relative to serum (equivalent to 18 p.c. haematocrit).
Ordinate: Optical density.
Abscissa: Stations of observation (edge of tube falls at 2).
Fig. 7. Effective optical path length. See text and Table 3.

As an additional check, the optical densities of a whole, and a haemolyzed sample of blood, relative to saline, were measured in turn at each station. The three fluids were introduced as described. The ratio of their densities was calculated at each station of observation. It was found that, from the third station inward, the ratios showed only a small decrease. The interpretation of the large error at the edge of the tube is suggested below.

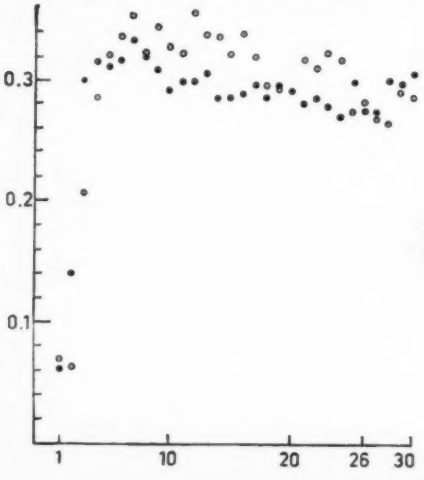

Fig. 8. See Table 3. Closed circles (b), open circles (a). Ordinate: Ratio A/B. Abscissa: Stations of observation (centre of tube at 26).

TABLE 3 (a).

Station	Optical density A Haemolyzed cells	B Intact cells	Ratio A/B
1	0·0223	0·0310	0·074
2	0·0195	0·3087	0·063
3	0·0806	0·3932	0·205
4	0·0976	0·3424	0·285
5	0·1091	0·3403	0·321
6	0·1084	0·3217	0·337
7	0·1249	0·3510	0·355
8	0·1146	0·3533	0·342
9	0·1380	0·3979	0·347
10	0·1347	0·4102	0·328
11	0·1429	0·4410	0·324
12	0·1601	0·4458	0·359
13	0·1591	0·4674	0·340
14	0·1688	0·4998	0·338
15	0·1715	0·5306	0·323
16	0·1806	0·5319	0·340
17	0·1951	0·6062	0·322
18	0·1960	0·6555	0·299
19	0·1929	0·6520	0·296
20	0·2000	0·6806	0·294
21	0·2112	0·6608	0·320
22	0·2052	0·6532	0·314
23	0·2236	0·6834	0·327
24	0·2187	0·6803	0·321
25	0·2050	0·7380	0·278
26	0·2141	0·7487	0·286
27	0·2023	0·7427	0·272
28	0·2023	0·7556	0·268
29	0·2175	0·7404	0·294
30	0·2131	0·7360	0·290

TABLE 3 (b).

Station	Optical density A Haemolyzed cells	B Intact cells	Ratio A/B
1	0·0235	0·3757	0·062
2	0·0593	0·3711	0·160
3	0·1205	0·3953	0·300
4	0·1803	0·3424	0·316
5	0·1102	0·3451	0·311
6	0·1202	0·3793	0·317
7	0·1353	0·4046	0·334
8	0·1388	0·4350	0·319
9	0·1451	0·4674	0·310
10	0·1451	0·4073	0·293
11	0·1562	0·5200	0·300
12	0·1602	0·5418	0·300
13	0·1761	0·5740	0·307
14	0·1761	0·6118	0·288

TABLE 3 (b)—Continued.

Station	Optical density A Haemolyzed cells	Optical density B Intact cells	Ratio A/B
15	0·1860	0·6484	0·287
16	0·1929	0·6628	0·291
17	0·2032	0·6803	0·299
18	0·2032	0·7038	0·289
19	0·2126	0·7132	0·298
20	0·2173	0·7427	0·293
21	0·2156	0·7597	0·284
22	0·2203	0·7644	0·288
23	0·2264	0·8062	0·281
24	0·2173	0·7970	0·273
25	0·2264	0·7518	0·301
26	0·2032	0·7286	0·279
27	0·2050	0·7380	0·278
28	0·2322	0·7652	0·303
29	0·2341	0·7781	0·300
30	0·2354	0·7600	0·308

DISCUSSION.

The very complex theory of micro-densitometry has been developed by Caspersson (1936) and Thorell (1947), for single, spherical objects of cellular dimensions, and has led to the conclusion that reliable measurements can be made only for as far as 0·85 of the radius of the object from the centre. It was therefore the aim of these initial experiments to determine to what extent this limitation applied to the present system. A further complication was anticipated from the use of a particulate suspension, and calculations were therefore made to determine to what extent the optical density of suspensions deviated from the Lambert-Beer law. The theoretical treatments of Rayleigh (1871, 1899) and Ryde (1931) could not be adapted to the present problem as they considered only spherical particles.

Suspensions of red cells, under certain conditions, show changes in light reflection and transmission (see Bayliss, 1952) due to orientation of the cells. Such changes will be the subject of subsequent communications, but to avoid complications in the initial experiments, sheep red cells were employed since it was found that such a system did not exhibit orientation effects. The low pressure gradient was used to minimize the formation of a marginal zone, and the assumption was made that the cells were evenly distributed over the cross-section of the tube.

The effective length of the light path through an homogeneous absorbing medium (haemolyzed blood), deviated only slightly from that expected in the case of a non-refracting body illuminated by parallel light where the graph of length v. distance of the point of observation from the centre of the tube should

be an arc of a circle (see Fig. 7). The optical densities, as measured, were therefore very nearly a true measure of the extinction coefficients of the specimens. The variations in the values for the various terms as calculated at points near the edge of the tube were probably attributable to two sources of error. The first, as mentioned above, was the lack of coincidence of the points of observation in separate runs; the second, variation in the reflection and refraction of the incident and emergent beams at the glass-liquid interface with the liquids of different refractive index. These variations were probably responsible for the wide descrepancies shown in Fig. 8 for the first four stations.

In the two experiments, set out in Table 3 and Fig. 8, it was found that the ratio of the optical density of a suspension of cells to that of a sample of haemolyzed blood remained almost constant from point to point across the tube, decreasing slightly, however, towards the centre. That is to say, for an identical optical path (the effects of refraction being small except close to the edge) the extinction coefficient of the suspension increased slightly with the length of the path, by about 10 p.c. for a 100 p.c. increase in path length. Calculating the ratios of densities given in columns 2 and 3 of Table 2 gave the values shown in column 4. These were rather more constant except from the 43rd station onwards, where lack of coincidence was probably responsible.

On the basis of these results it was possible to conclude that the apparatus would give reliable measurements of the optical density of the stream over 80–90 p.c. of its diameter, and that, although subject to error in magnitude, changes could be detected in the remaining region near the wall. In the experiments for which this apparatus was designed, measurements were made only of alterations in the optical density of the stream of blood with varying rates of flow, and so errors introduced in the experiments reported above, by the different refractive indices of the liquids employed, were avoided.

SUMMARY.

A description is given of an apparatus consisting of a regulated light source, a microscope, a scanning mirror and a photomultiplier tube, which enabled the measurement of changes in optical density of different regions of a stream of blood flowing in a narrow glass capillary tube. In this paper initial observations are reported, showing that the optical density of specimens of whole blood deviated slightly from the Lambert-Beer law, but that the apparatus could reliably detect changes in the constitution of the stream.

Acknowledgments.—We are indebted to Mr. R. Jones for his assistance with the construction of the apparatus in the workshop of the Department of Pathology; to Dr. S. G. Tomlin, of the Department of Physics, for the loan of the voltage regulator and the preparation of the mirrors; to Dr. J. E. McCartney, of the Institute of Medical and Veterinary Science for the supply of sheep's blood; and to other members of the Department of Pathology for assistance with the experiments.

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UROPORPHYRINS

III. CRYSTALLINE UROPORPHYRIN FROM NORMAL HUMAN URINE

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(Accepted for publication 7th April, 1954.)

Nicholas and Rimington (1949) have shown that normal urine contained traces of uroporphyrin, and Lockwood (1953) has shown that the daily excretion of uroporphyrin in a small series of normal urines was 15–30 μg. The present paper describes the isolation of sufficient uroporphyrin from the urine of two normal subjects to obtain the porphyrin in a crystalline state and to characterise its isomeric type by paper chromatography of the uroporphyrin itself, and by paper chromatography of the coproporphyrins obtained by decarboxylation.

METHODS.

Isolation.

In planning the isolation of the porphyrin from urine it was thought that 0·2 mg. of porphyrin would be sufficient for complete characterization and that it should be possible to avoid total losses greater than 50 p.c. No attempt was made to collect the total excretion of urine from the two subjects, most being collected between the hours of 9 a.m. and 5 p.m. The urine was treated in 4 litre batches.

The mixed uro- and copro-porphyrins were precipitated directly from the urine by adding 50 ml. of 40 p.c. $CaCl_2$ (w/v) per litre and then precipitating the calcium salts by the addition of 40 p.c. NaOH (w/v). At first NaOH was added until the first permanent precipitate, and then a further 30 ml. per litre. Later, it was found more satisfactory to add just sufficient alkali to bring the pH over 10 (test papers). The precipitate was then spun down, washed with methanol (500 ml. per litre of urine) and suspended in 250 ml. of methanol. The solution was approximately saturated with dry HCl. There was always a small insoluble residue at this stage, but this did not disturb the further treatment. In treating the first batches the acid methanolic solution was poured into a large volume of ice, water and chloroform and shaken vigorously. Pouring into chloroform, ice and sodium acetate solution was also tried, but the chloroform always gave troublesome emulsions. Finally, ether and sodium acetate were used and found satisfactory. The ether was well washed with water and a small amount of solid at the interface discarded. It was found unnecessary at this stage to wash with sodium carbonate. The small amount of purification achieved did not compensate for the loss of partially esterified porphyrins. In the first batches the solvent was evaporated off and the porphyrin was stored as the dry residue. This led, however, to the partial formation of the copper complexes, both of the copro- and of the uro-porphyrin esters. Some

[1]This work has been supported by grants from the National Health and Medical Research Council of Australia.

recovery was possible by reduction with sodium amalgam and allowing the porphyrinogens thus formed to reoxidise, but the loss was considerable. Purification was therefore carried a further stage before storing. The mixed porphyrin esters were saponified by extracting from an ethereal solution into 3N HCl and allowing to stand overnight. The copro-porphyrin was then removed by extraction with ether after just neutralizing to Congo red with sodium acetate solution. Phosphate (0·5 ml. of 0·2 M KH_2PO_4) and excess calcium (1 ml. 40 p.c. $CaCl_2$, w/v) were added and sufficient NaOH to bring the pH to 10. The precipitate was spun down, washed with methanol, and dissolved in dry methanolic hydrochloric acid. The solution was allowed to stand overnight so that esterification would be complete and the porphyrin was then taken into ether by neutralization with sodium acetate and rapid extraction. About 1 ml. of benzene was added and the ether was driven off on the water bath, a little more benzene being added to replace that lost while driving off the ether. This crude benzene solution of the porphyrin ester could be stored at −10° C. without the formation of any copper complex. The porphyrin ester could be crystallized at this stage, but the losses were less if chromatographed first. Chromatography on $MgCO_3$ was not satisfactory, but MgO gave very good results (Nicholas, 1951). The pigment was adsorbed from the benzene solution and the column was then washed with pure benzene. This eluted an oily impurity and some non-porphyrin pigments. The uroporphyrin ester was eluted with a 2 p.c. v/v solution of methanol in benzene. The porphyrin was crystallized from the eluate by replacing the benzene by methanol on the boiling water bath without allowing the vessel at any stage to evaporate completely to dryness. The porphyrin from 4 litres of urine crystallized from about 0·2 ml. of methanol.

Recrystallization.

The porphyrin ester was recrystallized by filtering off the crystals on a Willstätter knob from the methanol mother liquors and washing with a couple of drops of ether. The porphyrin was dissolved by pouring a few drops of warm chloroform onto the paper with gentle suction. The chloroform was then replaced by methanol on the boiling water bath as described above for benzene replacement.

Samples for melting point and microscopic examination were taken with a small capillary tube and transferred to a slide and washed with one or two drops of ether. The solubility of uro-porphyrin ester in methanol and ether is so low that there is no difficulty in recrystallizing amounts as small as 50 μg. several times.

However, repeated crystallization did not give satisfactory crystals, even after extraction of the ester from an ether solution (with a little chloroform to make the ester soluble) into hydrochloric acid, reprecipitation on calcium phosphate and esterification in order to remove possible traces of the copper or other metal complex. Recrystallization from ether was no better. The ester was finally crystallized satisfactorily by allowing a solution in about 0·2 ml. each of methanol and chloroform to evaporate slowly in a partially evacuated desiccator containing a dish of methanol and a dish of paraffin. The solution evaporated completely to dryness overnight and the residue was suspended in methanol. This gave good crystals (Fig. 1).

Saponification.

To obtain the free acid from the octamethyl ester for chromatography the porphyrin was extracted from an ether solution into 3 N HCl. The porphyrin was concentrated by standing it overnight in a desiccator containing both sulphuric acid and a small amount of potassium hydroxide. A further evacuation with potassium hydroxide completely removed the remaining hydrochloric acid. The porphyrin was then taken up in 2 p.c. ammonia.

Lutidine paper chromatography.

The pigment was subjected to lutidine paper chromatography in order to determine the number of carboxylic acid groups (Nicholas and Rimington, 1949; Kehl and Stich, 1951). An ascending chromatogram was run on Whatman's No. 1 paper in 20 p.c. ammonia vapour. A lutidine-water solution (60:40) was used.

Decarboxylation.

The uroporphyrins were decarboxylated by heating in 1 p.c. HCl at 180° C. (Fischer and Zerweck, 1924). The first attempts at decarboxylations failed to yield any detectable coproporphyrin and quite a lot of material was wasted in this way. The reason for this was not found, but may have been due to the prolonged (three hours) heating in the presence of small amounts of accompanying impurities.

Fig. 1. Micro-photograph of crystalline uroporphyrin from normal human urine.

Samples containing 5 μg. of crystalline "Waldenström" uroporphyrin gave an easily detectable amount of coproporphyrin, sufficient for a paper chromatogram of the ester. However, the following method was found satisfactory: a chloroform solution of the ester (about 10–20 μg. crystallized once after chromatography of the crude ester on MgO) was introduced into a small pyrex tube and allowed to evaporate slowly to dryness; it was then dissolved in methanolic hydrochloric acid and heated to drive off some of the methanol and hydrochloric acid; one drop of water was then added and the tube heated in the water bath for about 20 minutes to drive off most of the remaining methanol; the aqueous hydrochloric acid solution was then warmed and concentrated to about a fifth of its volume under vacuum—it was important not to bring completely to dryness, as the residue was difficultly soluble in 1 p.c. hydrochloric acid; the tube was then heated for 80–90 minutes at 175–180° C. In preliminary experiments with "Waldenström" uroporphyrin (5–25 μg.) no ether insoluble porphyrin remained after heating for this time and the ether soluble porphyrin produced showed an absorption maximum in N HCl at 402 mμ, characteristic of coproporphyrin, and no trace of a shoulder at 407 (the position of the Soret band of uroporphyrin in N HCl).

The work of Edmondson and Schwartz (1953) on optimum conditions for the decarboxylation of uroporphyrin appeared, unfortunately after the completion of this work; we might otherwise have been spared the loss of much valuable material. For very small quanti-

ties, however, it may be useful to use the method of solution in hydrochloric acid described above as well as the reduction of oxygen tension advocated by Edmondson and Schwartz. We found in preliminary tests, using 10 μg. of authentic uroporphyrin, if 1 p.c. HCl was added to the dried residue, either of the ester from chloroform solution or of the dry hydrochloride from hydrochloric acid solution, that the yield of coproporphyrin was considerably reduced.

After decarboxylation the porphyrin was esterified in the normal way and evaporated to dryness from an ether solution. The residue was taken up in acetone.

Kerosene paper chromatography.

To distinguish between coproporphyrin I and III, an ascending paper chromatogram was run according to Chu, Green and Chu (1951) in kerosene/chloroform (chloroform vapour) followed by kerosene/propanol (kerosene vapour).

RESULTS.

Treatment of 50 litres of urine from one subject (W.H.L.) yielded, after losses described under Methods, 100 μg. of crystallized uroporphyrin. It was recrystallized five times. Fig. 1 shows the crystals after the fifth recrystallization. From 2 litres of urine from the second subject (B.B.) 9 μg. were obtained.

TABLE 1.

Melting points (uncorr.) of uroporphyrin ester from normal human urine.

	W.H.L.	B.B.
1st crystallization	277–284° C.	
2nd recrystallization	292–296° C.	295–305° C.
3rd recrystallization	292–296° C.	
4th recrystallization	295° C.	
5th recrystallization	295–305° C.	

Melting points. Table 1 shows the melting points (uncorr.) obtained on a Griffin and Tatlock micro melting point apparatus with a curved thermometer embedded in a copper block. These indicate uroporphyrin I with less than 25 p.c. of uroporphyrin III (Nicholas and Rimington, 1953).

TABLE 2.

Visible spectrum in mμ.

Waldenström uroporphyrin (M.P. 248–253° C.)	625·4	571·2	534·4	505·8
W.H.L.	624·4	570·2	535·0	503·3
B.B.	623·6	571·6	534·8	—

Visible spectrum. Table 2 gives the visible spectrum of the uroporphyrins in chloroform solution measured on a Beck-Hartridge reversion spectroscope.

Lutidine paper chromatography. Four spots were run: the uroporphyrin crystallized once after chromatographing the crude preparation on MgO; the

porphyrin in the mother liquors from which these were obtained; "Waldenström" uroporphyrin (M.P. 248–253° C.); coproporphyrin III (M.P. 145° C. then 156–158° C.). The crystallized porphyrins from both subjects showed a single spot with the same Rf as an authentic specimen of uroporphyrin on the lutidine paper chromatogram. The porphyrin from the mother liquors consisted mainly of a porphyrin with the same Rf value, but showed a second, weaker, spot with an Rf value close to that of coproporphyrin. The chromatogram for the porphyrins from the first subject (W.H.L.) is illustrated in Fig. 2.

Fig. 2.

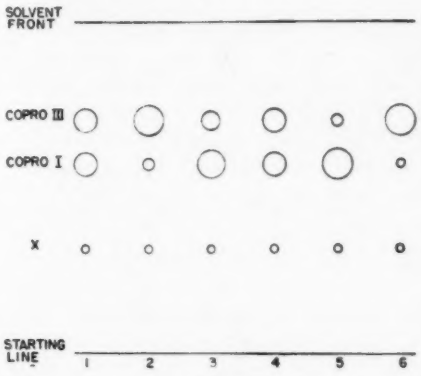

Fig. 3.

Fig. 2. Lutidine paper chromatogram of uroporphyrin from normal human urine. 1. Coproporphyrin III (M.P. 145° C. then 156–158° C.). 2. Porphyrin from mother liquor of crystalline urinary uroporphyrin (W.H.L.). 3. Crystalline urinary uroporphyrin (W.H.L.). 4. Authentic uroporphyrin (M.P. 248–253° C.).

Fig. 3. Diagram of kerosene paper chromatogram of coproporphyrins obtained by decarboxylation of uroporphyrin of normal urine.

1. Coproporphyrin ester from "Waldenström" uroporphyrin (M.P. 248–253° C.). 2. Coproporphyrin ester III (M.P. 145° C. then 156–158° C.). 3. Coproporphyrin ester from B.B. uroporphyrin. 4. Urinary B.B. coproporphyrin ester. 5. Coproporphyrin ester from W.H.L. uroporphyrin. 6. Urinary W.H.L. coproporphyrin ester. Relative strengths estimated by inspection are indicated by the area of the circles.

Kerosene paper chromatography. Four spots were run: the porphyrin produced by decarboxylation of the urinary uroporphyrin; the urinary coproporphyrin from the same subject; the coproporphyrin produced by decarboxylation of crystalline "Waldenström" uroporphyrin (M.P. 248–253° C.); crystalline coproporphyrin III (M.P. 145° C. then 156–158° C.).

The esters of the decarboxylation products of the crystallized porphyrins from both subjects gave two spots, corresponding to coproporphyrins I and III, on the kerosene paper chromatogram (see Fig. 3). The porphyrins from both subjects gave preponderantly coproporphyrin I. W.H.L. porphyrin contained

only a trace of isomer III; B.B. porphyrin contained more, but still considerably less than 50 p.c. It is interesting to note that the relative abundance of the isomers is reversed in the urinary coproporphyrins: B.B. coproporphyrin contains approximately equal amounts of each isomer; W.H.L. coproporphyrin contained very little of isomer I. In Fig. 3 the relative strength, as judged by inspection, is indicated by the size of the spots. All specimens showed a third spot, marked "x" in Fig. 3, which was not further studied.

DISCUSSION.

It is possible that the difference found between the relative abundance of the isomers in urinary copro- and uro-porphyrins arises either from preferential loss of uroporphyrin III in MgO chromatography, in crystallization or from preferential destruction of the III isomer during decarboxylation, but the difficulties experienced in separating the two uroporphyrin isomers make it much more probable that the difference is real.

It is interesting that, of the four naturally occurring porphyrins, uroporphyrins I and III and coproporphyrins I and III, only uroporphyrin III appears to be virtually absent from normal urine. This can well fit the hypothesis (although it can hardly be used as evidence for it) that of the four it is the only porphyrin that is on the direct path in the formation of haemoglobin.

SUMMARY.

Uroporphyrin has been isolated and crystallized from the urine of two normal subjects and proved by melting point, and chromatography of the derived coproporphyrins, to be uroporphyrin I with a trace of uroporphyrin III.

Techniques for crystallizing and decarboxylating small amounts of uroporphyrin down to 10 μg. are described in detail.

Acknowledgment.—The authors are indebted to Dr. R. Lemberg, F.R.S., for help and encouragement during this work.

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